PHENOTYPIC AND GENOTYPIC VARIATIONS IN FLORAL TRAITS THAT AFFECT MATING SYSTEM OF *COLLINSIA HETEROPHYLLA*

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ABSTRACT

This research studied phenotypic and genetic variations in eight floral morphological traits that affect the mating systems of *Collinsia heterophylla,* effects of pollination treatments on flower longevity and pollen-tube growth rate. The aim was to assess how phenotypic and genetic variations in floral traits, as well as other interacting mechanisms, influence mating systems in plants. This study found that, floral traits varied continuously within- and between-populations, and across floral developmental stages in Norway and Chiltern populations. Floral traits showed high correlations and heritabilities, with corolla, stamen and pistil recording the highest correlations in the two populations. Thereby, suggesting genetic linkages or pleiotropy effects among traits. Consequently, traits either evolve together or the selection of one trait constrains the other. Thus, the termination of pistil life through pollination and fertilisation could impact on structure and functions of the corolla, pistil and the stamens. The study also found that, the effects of pollination treatments, time of pollen arrival and pollination significantly affect flower longevity. Furthermore, autonomous selfing occurred early in Norway population, but late in Chiltern population. Inter-population cross pollination treatment showed shortest flower longevity in Norway population than Chiltern, indicating differential pollen-tube growth rate. Pollen-tube growth rate was assessed *in-vitro and in-vivo* and results showed no correlation in pollen-tube growth rate *in-vitro* and *in-vivo.* However, Chiltern population had longer pollen-tube growth *in-vivo* than Norway population. Similarly, Chiltern population grew longer pollen-tubes on the Norway style than on the Chiltern and vice versa in Norway pollen-tubes; suggesting partial cryptic self-incompatibility (CSI) in *C. heterophylla.* Therefore, floral traits variations, correlations, heritabilities, flower longevity and post-pollination processes can drive the course of mating systems evolution in flowering plants.

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AUTHOR'S DECLARATION

I hereby declare that whilst registered as a candidate for the award of Doctor of Philosophy, I have not been registered for any other research award. The results and conclusions presented in this thesis are my own and have not been presented previously or separately for any other award.

Signed …… Date……

Maria Bolatito Oyegbile

GENERAL OVERVIEW, ORGANISATION AND LAYOUT OF THESIS

This thesis was prepared using the Bookman Old Style MS character with Harvard referencing style. The thesis is divided into six chapters. As a whole, the work presented gives account of the research carried out to investigate the phenotypic and genotypic variations in floral traits that influence the mating systems of *Collinsia heterophylla*.

In chapter one, an overview of the past and current literature on mating system evolution in plants is presented. A closer look is taken at the different types of mating systems in angiosperms and what mechanisms have been attributed to the evolution of these mating systems. The concept of mating system is briefly introduced, followed by the types of mating system and the evolutionary models proposed for the evolution of mating systems. This chapter also looks at the floral traits and their effects on mating system types as well as the ability of these traits to be pass from one generation to the other.

Chapter two describes the study plant extensively and gives an overview of the experimental procedure and design of all experiments carried out. Chapter three to five are result chapters and each of these chapters is presented in the form of a detailed scientific publication and is therefore divided into its own introduction/literature review, materials and methods sections, the actual results followed by a discussion and brief conclusion. The final chapter discusses the findings of the investigation as a whole, followed by the reference section and appendices.

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LIST OF ABBREVIATIONS

ASC	Anther Stigma Contact
ANOVA	Analysis of Variance
CSI	Cryptic Self-incompatibility
H^2	Heritability
SD	Standard Deviation
SE	Standard Error
SPSS	Statistical Packages for Social Sciences
WISO	Weak In-breeder/Strong Out-breeder
PGR	Plant Growth Regulators

ACKNOWLEDGEMENTS

The work presented in this thesis is original. The experimental work has been carried out by the author. The result tables and figures have been generated by the author from the research work carried out.

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DISSEMINATION

Post-pollination mechanisms in *Collinsia heterophylla*: pollen-tube growth rate. A paper presented at the 54th Annual Meeting of the Ecological Genetics Group, 6th-8th April 2010 The University of Stirling, UK.

DEDICATION

I dedicate this thesis to the memory of my Late Dad Emmanuel Oladele Akanji and my Late Father in-law Pa Solomon Ajiboye Oyegbile.

PHENOTYPIC AND GENOTYPIC VARIATIONS IN FLORAL TRAITS THAT AFFECT MATING SYSTEMS OF *COLLINSIA HETEROPHYLLA*

CHAPTER 1

1.0 General Introduction

1.1 Background

The world's quarter of a million vascular plant species display a remarkable diversity of life histories, growth forms and physiologies; but the diversity and complexity of their reproductive system is even greater (Barrett, *et al.,* 1996; Holsinger, 2000). It has been observed that flowers generally vary greatly in size and shape. The effects of this variation in floral traits on plant mating systems have been the focus of both theoretical and emperical studies for more than a century now, since the time of Darwin (Darwin, 1877, 1878; reviewed in Armbruster *et al.*, 2002). In crop breeding and improvement, the knowledge of the mating systems and the factors that affect the mating systems of crop plants is very important. It has been reported that mating systems (that is the possibility of outcrossing and selfing) can be affected by floral-trait variation (see Fenster & Ritland, 1994; Holtsford, 1996; Johnston & Schoen, 1996; Holtsford & Ellstrand, 1997; Rausher & Chang, 1999). Studies of phenotypic variation in size and shape of floral traits, as well as the inheritance of such floral trait are important, because the mating system defines how genes are passed from one generation to the next and, in essence, controls the evolution of all other traits of the organism (Kalisz *et al.*, 2001).

A number of researchers have reported frequent shifts from outcrossing to inbreeding (Stebbins, 1974; Grant, 1981; see Barrett *et al.*, 1996; Ramsey and Schemske 1998; Armbruster *et al.*, 2002; Tate, 2002; Shimizu et al., 2004; Kubota and Ohara, 2009). This evolutionary transition was described as the "well-trod evolutionary pathway" by Stebbins in 1974. This shift in mating systems from outcrossing to selfing has been observed to involve a change in a large number of floral developmental and morphological traits; for example, it is generally known

that, when a species shifts from outcrossing to selfing, the sizes of the flower and flower parts become reduced and floral development is shortened (e.g. Lloyd 1965; Arroyo 1973; French *et al.*, 2005). A similar result was obtained by Tate and Simpson (2004), who reported that, in *Tarasa* species, a shift from outcrossing to inbreeding had repeatedly involved an overall reduction of floral morphology that includes smaller petals, fewer anthers per flower and fewer pollen grains in each anther. They observed that pollen/ovule ratio between the diploids and tetraploids of *Tarasa* were striking [2*n*=20 tetraploid (5-20) and the 2*n*=10 diploid (24-102)]. Cruden (1977) also reported earlier a decrease in the quantity of pollen produced in relation to the number of ovules in inbreeding species (see Cruden, 2000; but see also Wyatt, et al. 2000). Other floral traits have also been similarly influenced by transitions in mating systems; for example, shifts in the arrangement and lengths of stamens and styles, so that the spatial separation between anthers and stigmas is very much reduced in selfers (e.g. *Datura stramonium* (Solanaceae); see Lyons *et al.*, 1989; Motten and Antonovics 1992; Motten and Stone 2000).

In flowering plants, the floral phenotype, that is, the characteristics of individual flowers including their size, structure, sexual condition, colour, scent, nectar production and degree of herkogamy and dichogamy, has been seen to influence the type of mating system exhibited by a species or population. Outcrossing species have been observed to have large flowers with large corollas, large anthers, abundant pollen, numerous ovules, late style elongation, late stigma receptivity, and late anther-stigma contact (self-pollination). In contrast, inbreeding species have been observed to have reduced flowers with small corollas, small anthers, less pollen, fewer ovules, early stigma receptivity, and early anther-stigma contact (Motten and Stone, 2000; Armbruster et al., 2002; Goodwillie and Ness, 2005; Takebayashi et al., 2006; Lankinen et al., 2007). This type of variation has also been reported within populations by Sinervo and Svensson (2002). Busch (2005) suggested that this variation within species and populations has a genetic basis and may be the result

of inconsistent selection for reproductive assurance in the absence of pollinators.

Although many studies and theoretical models have predicted extreme selfing or outcrossing in the majority of plants species (Lande and Schemske, 1985; Barrett, 2010), recent theoretical and experimental studies propose that there is a variety of mating-system types across species (Goodwillie et al., 2005). Intermediate or mixed mating systems have been extensively reported among most species of *Collinsia* and *Tonella* (Plantaginaceae; Vogler and Kalisz, 2001; Armbruster *et al.,* 2002). However, it is still not clear if mating systems and the developmental, as well as morphological traits associated with mating systems vary continuously across populations or if these are clearly classified into two extreme classes (Armbruster, 2006).

Several studies have shown that there is lack of integration in floral parts (traits) and hence can occasionally evolve independently (Young *et al.* 1994; Fenster *et al.*, 1995; Delph *et al.*, 1997; Bixby & Levin 1996, Eckhart 1999; Runions & Geber, 2000); but some researchers have observed that functionally related traits often co-vary within and among populations and species (e.g. Endler, 1984 and 1986; Conner and Via, 1993; Mazer and Hultgard, 1993; Armbruster and Schwaegerle, 1996; Conner, 2002; Sinervo and Svensson, 2002). The genus *Collinsia* has been reported by Armbruster *et al.* (2002) to show strong among-species correlations between (interlaid) flower size, anther size, pollen number, timing of floral part elongation, timing of stigma receptivity, and mating system (inbreeding vs. outcrossing).

The principal objective of this study is to investigate the phenotypic and genotypic variations in flowers of two populations of *Collinsia heterophylla* species (obtained from two different geographical locations); and to assess how the genetics of floral traits in these two populations influence the evolution of the mating systems in this plant species. This study examines the basic issues of phenotypic and genetic correlations in eight floral traits associated with mating systems, as well as their heritabilities and evolvabilities. Furthermore, this study attempts to

answer the questions of whether floral-trait variations in these two populations will influence floral longevity as well as pollen-tube growth in this plant.

1.2 Introduction

In a review of the evolution of plant sexual diversity, Barrett (2002) stated that flowers, the reproductive organs in angiosperms, are more varied than the equivalent structures of any other group of organisms. That is why a centre of theoretical and empirical studies, since the time of Darwin's monographs on plant sexual systems (Darwin, 1877, 1878), has been the diversity of floral traits in angiosperms and their effects on plant mating systems (Armbruster *et al.*, 2002). This type of floral traits variation within and among species has been found to influence the type of mating systems exhibited by plants, whether outcrossing or selfing (Mazer and Hultgard, 1993; Holtsford, 1996; Johnston and Schoen, 1996; Holtsford and Ellstrand, 1992; Fenster and Ritland, 1994; Herrera, 2001; Mazer and Delesalle, 2002; Parachnowitsch and Elle, 2004).

1.2.1 Mating Systems in Plants

Mating systems (that is, the average frequencies of cross- versus self-fertilisation) in plants are very important, because they have effect on the spatial and temporal patterns of genetic diversity within and between populations. Barrett and Harder (1996) stressed that the mating system determines how genes and traits are transmitted from parent to offspring across generations (see also Barrett, 2003). Mating system is believed to be greatly influenced by various factors such as immobility, hermaphroditism and dependence on vectors for transporting pollen (Barrett and Harder, 1996).

Most plants are outbreeders, promoting cross-ferilisation by a variety of genetic, developmental, and morphogenetical mechanisms (Simmonds, 1979; Simmonds and Smartt, 1995 & 1999; Bos and Caligari, 2008). Outbreeding promotes heterozygosity, high recombination rates, evolutionary flexibility, and the opportunity to export favourable

recombinants. Inbreeding, on the other hand, is a condition that most likely evolved from outbreeding. In contrast to outbreeding, inbreeding promotes homozygous leading to the elimination of deleterious recessive allels (see Holsinger, 2000). Floral traits have been observed to have a considerable effect on the probabilities of outcrossing vs. inbreeding (Fenster and Ritland, 1994). According to Barrett (2002), relatively large flowers and large temporal or spatial separation of anthers and stigmas generally promote outbreeding, while self-pollination is usually associated with small flowers and little temporal or spatial separation of anthers and stigmas.

1.2.2 Mating-System Evolution

A major feature of angiosperm evolution is the repeated, parallel, evolutionary changes in mating systems (Stebbins, 1974; Armbruster *et al.*, 2002). Flowering plants have been observed to exhibit more complex mating patterns than most animal groups. For instance, individual parent plants frequently mate with numerous mating partners, as well as with themselves, simultaneously during their reproductive lifetime (Barrett, 1985). Most plants acquire this ability from their hermaphroditic nature, their dependence on biotic and abiotic vectors for pollination and dispersal as well as, the fact that gender in plants varies in a quantitative manner depending on the mating system, demography and life-history strategy of populations (Barrett, 1985; Barrett, 2003; Steets, *et al.*, 2007). Mating systems are thought to influence population genetics and population evolution through their effects on actual population size and effective recombination rate (Glémin, 2007).

Mating-system evolution in plants has been the subject of numerous studies over the past few decades (Georgiady *et al.*, 2002). Generally, theoretical models of mating-system evolution have focused on the fitness consequences of selfing and outcrossing. This has inspired substantial empirical work on the ecology and genetics of both inbreeding depression (Barrett and Harder, 1996; Kittelson and Maron, 2000) and outbreeding depression (Peer and Taborsky, 2005). These studies have

either used quantitative-genetic techniques or have dealt exclusively with morphology and development. Bixby and Levin (1996) have reported a few studies on evolvability of mating systems in wild plants; however, little work has been done on the control of quantitative characters involved in flower development (Georgiady *et al.*, 2002).

1.2.3 Outcrossing

In most angiosperms, outcrossing is expected to be a normal process of mating and this is usually imposed by self incompatibility (Crowe, 1964; Brandvain and Haig, 2005). Consequently, in order to carry out successful crossing, most plants employ a variety of genetic and morphogenetic mechanisms (Simmonds, 1979; Simmonds and Smartt, 1995 & 1999). Outbreeding is generally promoted by relatively large flowers and temporal or spatial separation of anthers and stigmas (Barrett, 2002). It is usually favoured by high rates of pollinator visitation even in populations of self-compatible plants, especially when selfing is delayed (Simmonds, 1979; Simmonds and Smartt, 1995). Outbreeding frequently promotes heterozygosity, which over the short term carries a high load of deleterious recessive genes that are responsible for the inbreeding depression; however, over the long term this allows persistence of high genetic load.

Outcrossing also promotes high recombination rates, evolutionary flexibility, and the opportunity to export favourable recombinants. Hence, outcrossers have more genotypes within population than selfers and asexual, which have less genetic variation among individuals within a population. But selfers and asexuals often have more genetic variation across different populations than sexual outcrossers. This is because the former experience less gene flow than the latter as a result of the mating system (Holsinger, 2000).

1.2.4 Inbreeding

Inbreeding has evolved repeatedly in angiosperms, although little is known about the developmental and genetic processes involved (Georgiady

et al., 2002). Generally, inbreeding refers to any mating system which involves fewer ancestors than random mating (outbreeding); it is generally thought to have evolved from outbreeding (Simmonds, 1979; Simmonds and Smartt, 1995 & 1999). Inbreeding exposes genetic variation to the effects of natural and artificial selection; thus it plays an important role in the genetic improvement of crops (Sharma *et al.*, 1996).

Self-pollination or selfing is an extreme form of inbreeding; it frequently occurs within a flower without the aid of a pollen vector (Simmonds, 1979; Simmonds and Smartt, 1999). Lloyd (1992) proposed that, over evolutionary time, prior self-pollination may lead to earlier and more complete seed set, because resources can be directed to seed and fruit production instead of pollinator attraction through large and long-lived flowers. Thus, prior self-pollination is advantageous if pollinator visitation rates are low; but will always yield high levels of inbreeding (see also Sharma *et al.*, 1996).

It has been argued that pollinator scarcity and reproductive assurance are likely to be important drivers of plant mating system evolution. For example, reproductive assurance has been frequently employed as an explanation for the evolution of self-fertilisation in plants (Moeller, 2005). Although the ability to spontaneously self-fertilise when mates or pollinators are in short supply may ensure reproduction (Darwin, 1877; Lloyd, 1980; Holsinger, 1996); its value is degraded if pollen or ovules that would have been involved in outcrossing are impeded. This is referred to as pollen and ovule discounting respectively (Lloyd, 1992; Holsinger, 1996; Herlihy and Eckert, 2002; reviewed in Weekley and Brothers, 2006). Moreover, Fisher (1941) has previously reported that selfing is capable of giving rise to less fit progeny; this is commonly referred to as inbreeding depression.

1.2.5 Inbreeding Depression

According to Charlesworth and Charlesworth (1987), inbreeding depression can be defined as the loss of fitness arising from self-fertilisation or mating between relatives (Husband and Schemske, 1996).

It is considered as the principal force that counteracts the transmission advantage associated with selfing alleles (Darwin, 1877; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1988; Charlesworth, 2003).

Inbreeding does not only leads to a direct decline in fitness (that is, lower fecundity and/or survival (Dudash, 1990), but it may also affect characters such as flower size, flower symmetry and mating system (Karolyn, 1994; Andersson, 1996, 1997; Rao *et al.*, 2002,), which in turn can affect reproductive fitness. However, inbreeding depression in natural populations with a long history of inbreeding may not be strong since the genes causing this effect will have previously been removed ("genetic purging"; Charlesworth, 1988). Inbreeding has been reported by (Simmonds and Smartt, 1995 & 1999) to influence quantitative (metric) traits such as, yield, height and variability more than metric traits such as, floral parts, number of internodes or leaflets, as well as qualitative traits, (e.g. colour, shape and size of flowers and seeds). Consequently, inbreeding depression is a distinct feature of quantitative traits as opposed to qualitative traits (Núñez-Farfán *et al.*, 1996; Ashman and Majetic, 2006; Lankinen *et al.*, 2009).

Inbreeding depression has been observed to be more pronounced in outbreeding crops while it is greatly reduced in inbreeding crops (Simmonds and Smartt, 1999; but see also Sheridan and Karowe, 2000; Zajitschek *et al.*, 2009). However, Simmonds and Smartt (1999) have previously suggested that, regardless of the mating system of a crop plant, the crop's survival depends on the system of agriculture. Inbreeding depression is not a permanent state, and the genetic basis may fluctuate across different life stages (Husband and Schemske, 1995, 1996; Hedrick and Kalinowski, 2000; Ronce et al., 2009). In a review of inbreeding depression in plants, Husband and Schemske (1996) found that mainly outcrossing populations had larger inbreeding depression values for seed production, germination, and survival, but had comparable values to mostly self-fertilising populations for growth and flower production. In

addition, they pointed out that inbreeding is expected to change as a consequence of inbreeding history with populations that normally self-fertilise and have a lower level of inbreeding depression (see also Lande and Schemske, 1985). For instance, Latta and Ritland (1994) discovered a negative relationship between prior inbreeding and the extent of inbreeding depression for five fitness traits in populations of *Mimulus* species (Scrophulariaceae).

1.3 Factors Influencing Mating Systems in Plants

1.3.1 Floral Morphological Traits

Flowers are highly organised structures found in angiosperms. Wilson (1995) reviewed several research findings and emphasised that flower morphology can be described as being often complicated. This is because each floral part is in a specific location and can display colour patterns, spurs, pockets, or flanges that supposedly attract pollinators. These then channel the pollinators past the anthers and stigmas, where pollen is sent out and received, respectively. This process has often been thought to be rigorous and precise (Muller, 1883; Straw, 1956; Percival and Morgan, 1965; Macior, 1967; Beattie, 1971; Brantjes, 1982; Wolf and Stiles, 1989; Armbruster *et al.*, 1994).

According to Wilson (1995), selection on flowers has frequently been viewed as being particularly stringent, steady, and responsible for species differences. He noted that within species, flowers are reasonably constant and are relatively unaffected by environmental and developmental circumstances. However, several studies have identified great variation in flowers of some plant species; for example, *Collinsia* species (see Armbruster *et al.*, 2002). These flower variations have been suggested to affect mating system patterns in plants. The elucidation of relationships between specific floral traits and mating system will significantly enhance our understanding of mating-system evolution (Lankinen *et al.*, 2007). In species of *Collinsia* (Plantaginaceae), there is a broad variation in mating systems. This variation has been found to be associated with variation in

floral morphology and development, as well as with the timing of self pollination (Lankinen *et al.*, 2007).

1.3.2 Anther-Stigma Contact

Generally, the degree of self pollination within flowers has been found to be affected by two types of floral morphological traits. The first is the proximity of the male (anthers) and the female (stigmas) parts; this determines how easily self pollen can be deposited on the stigma (Kalisz *et al.*, 1999). Previous studies have shown that the distance between the anthers and the stigmatic surface (herkogamy), is positively correlated with outcrossing rates (e.g. Holtsford and Ellstrand, 1992; Belaoussoff and Shore, 1995; Karron *et al.*, 1997; reviewed in Kalisz *et al.*, 1999). The second is the difference in the time of the expression of the male and female phases (dichotomy). The longer the time interval between the male and female phases the lower the expected selfing rate (Bertin and Newman, 1993; reviewed in Kalisz *et al.*, 1999). However, when there is an overlap between the timing of the male and female phases or changes in the relative positions of the anther and the stigma during development, delayed selfing can occur; and this is achieved by different plants in various ways (Kalisz *et al.*, 1999; Runions and Geber, 2000; Marshall et al., 2010).

1.3.3 Pollen Viability and Pollen-Tube Growth

Since the inception of plant mating systems studies with Darwin's contributions in 1876 and 1877, the main focus of the studies has been on understanding the reasons for, and outcomes of, differences in self-pollination (selfing) and cross-pollination (outcrossing), rather than on aspects of male fertility and plant paternity. This was as a result of lack of understanding of the importance of the male factor of reproductive fitness as well as technical difficulties associated with studying mate diversity in plant populations (Barrett, 1985).

In angiosperms, pollen plays a vital role in the flow of genes from one generation to the other. Therefore, the quantity and quality of pollen

produced by a flower is an important component of fitness (Kelly *et al.*, 2002). Consequently, the study of pollen viability and the factors that enhance the successful delivery of the male nuclei to the female ovary is very essential in the understanding of mating system evolution. Pollen viability can be explained as a test of the ability of the pollen grain to store and protect the sperm nuclei until they fuse with the female nuclei. However, the pollen grain needs to develop a pollen-tube to transport the male nuclei from the stigma to the ovary, where they fuse with the egg and polar nuclei in the ovule. Thus pollen-tube growth rate is significant as it is an indication of the ability of the pollen grain to perform the function of delivering the sperm cell to the embryo sac during pollination.

Several methods have been employed to estimate pollen viability, for example, seed set data; but these data basically demonstrate the presence or absence of fertile pollen, or at most, provide the relative number or percentage of viable pollen among treatments. The true level of pollen viability cannot be determined with these data (Fei and Nelson 2003). The common method usually used to assess both pollen load and pollen viability is staining and direct counting (see Stanley and Linskens, 1974; Heslop-Harrison, and Shivanna, 1984; Barrett, 1985; Dudash, 1991; Willis, 1999). This method employs a range of staining techniques, for example, aniline blue for detection of the callose in the pollen walls and pollen tubes, iodine to determine starch content, iodine, or 1, 2, 3-triphenyl tetrazolium chloride (Brooking, 1979; Heslop-Harrison *et al.*, 1984; Mulugeta *et al.*, 1994; reviewed in Fei and Nelson, 2003); however, Mulugeta *et al.*, 1994 noted that pollen staining and viability are not always positively correlated.

More reliable tests of pollen viability are the *in-vitro* and *in-vivo* germination tests (Adhikari and Campbell, 1998; Montaner *et al.*, 2003). The *in-vivo* pollen germination test is done by observing pollen germination on a receptive stigma of the same flower, plant or species, provided there is no self incompatibility. In contrast, the *in-vitro* pollen germination involves growing pollen in an artificial medium. The medium used depends on the plant being investigated, but the three most common

components used when formulating an artificial medium for *in-vitro* pollen germination are sucrose (Bair and Loomis, 1941; DeBruyn, 1966a, b), H_3BO_3 (DeBruyn, 1966a, b), and calcium ions (Cook and Walden, 1967; reviewed in Fei and Nelson, 2003). There is currently very little information on which method is best for different crops. However, using information from different tests that have been carried out on other crops can help refine methods.

1.3.4 Stigma Receptivity

Stigma development from bud stage through anthesis and fruit formation is vital to the success of any plant population irrespective of its mating system pattern. The timing of stigma receptivity determines whether or not a plant species will be subject to self- or cross-pollination. The knowledge of this timing determines the fruiting outcome of a particular plant species in the presence or absence of pollinators. In order to understand how the timing of stigma receptivity is related to mating-system evolution, it is essential to carry out a detailed study of both timing of stigma receptivity and self-pollination simultaneously (Lankinen *et al.*, 2007).

1.3.5 Fertilisation-Induced Flower Senescence

Flower senescence is a process whereby petals and sepals disintegrate and close up. Flower senescence in many angiosperms is initiated by fertilisation (Weber and Goodwillie, 2007). However, this stage of development in some species of angiosperms has been considered to be associated with some hormonal responses; and the hormone believed to be responsible for flower senescence is ethylene (Stead, 1992; VanDoorn, 2002; Rogers, 2006). Weber and Goodwillie (2007) have observed that flower senescence is not only caused by hormonal response, but also caused by fertilisation. According to Ashman (2004), early pollination leads to early fertilisation and then possibly early senescence; therefore, early hand pollination may lead to shorter flowering time. When this occurs, maintenance cost is reduced thereby leading to greater investment

in seeds (Ashman and Schoen 1997). Consequently, the addition of pollen may alter the efficiency at which plants can convert ovules to seeds (Ashman *et al.*, 2004). It is thought that fertilisation-induced flower senescence has potential outcomes for the evolution of mating systems; for example, when autonomous self-pollination occurs it causes fertilisation and the flower senesces early. This indicates that flowers may senesce before all the available pollen grains have disseminated, thereby discounting unused pollen (Weber and Goodwillie, 2007).

When flower senescence is induced by fertilisation, there is a potential effect on mating-system evolution, because autonomous self-pollination (selfing that occurs in the absence of a vector) decouples the rates of fitness accumulation through ovule fertilisation and pollen dispersal (reviewed in Weber and Goodwillie, 2007). Hence, when fertilisation is entirely pollinator-mediated (e.g. in an obligate outcrossing hermaphrodite species), both male and female components of fitness depend on the action of the same pollen vector. Therefore, fitness through ovule fertilisation and pollen dispersal must be correlated to some extent, although the accumulation rates may differ (Ashman and Schoen, 1994; Schoen and Ashman, 1995; reviewed in Weber and Goodwillie, 2007). Sato (2002), noted that the effect of self-fertilization on flower senescence has received little or no attention, and, in addition, its effect on the evolution of selfing rates has rarely been measured judiciously (Weber and Goodwillie, 2007). As a result, in the chapter four of this thesis, the effect of four pollination treatments is assessed in order to understand how these two populations of *Collinsia heterophylla* will respond under the different pollination treatments.

1.4 Heritability

Heritability refers to the proportion of variation between individuals in a population that is determined by genetic factors. Therefore, heritability (H^2) can be defined as the amount of resemblance among relatives that is due to shared genes (Gurevitch *et al.*, 2002). It is the proportion of all the variation of a quantitative trait that is present as a

result of genetic variation in a population. It is important to note that heritability describes the population, not individuals within that population. According to McKay and Latta (2002) heritability is the main measure of genetic variations in polygenic (quantitative) traits. It can be measured as the slope of a regression of offspring traits values on parental traits values. Another common way to measure heritability is to measure the correlation among siblings, the slope of which is the measure of heritability (Gurevitch *et al.*, 2002).

The concept of heritability and its definition as an estimable, dimensionless population parameter was introduced by Sewall Wright and Ronald Fisher almost a century ago (Visscher *et al.,* 2008). Heritabilities of several characters have been measured by examining parent-offspring relationship. It was observed that there were significant heritabilities for timing of flowering, flower number, and branch number in three of four populations of *Collinsia heterophylla* studied by Charlesworth & Mayer (1995) (see Armbruster *et al.*, 2002). Other methods have also been suggested for estimating heritability, these include; mixed-model analysis of un-branched data (McLean *et al.,* 1991; Searle *et al.,* 1992 and Littell *et al.,* 1996), pedigree analysis (Xu, 2003), and use of DNA markers to estimate genetic components of variation (Ritland, 2000). However, Holland *et al.*, (2002) have argued that although the use of mixed models has been reported for animal breeding, its application to plant breeding has not been reviewed.

Heritability can also be estimated by performing artificial selection experiment. However, in order for evolutionary biologists to predict the phenotypic response to selection on mating systems and other characters, the knowledge of the strength of selection and the heritability or evolvability *(I_A)* (the capacity of a population of organisms to generate diversity and evolve through natural selection) of the characters is vital (Kirschner and Gerhart, 1998). In addition, Visscher *et al.* (2008) noted that heritability allows for an assessment of the relative importance of genes and environment to the variation of traits within and across

populations; and that heritability continues to be used to explain response to selection in evolutionary biology and agriculture, and predict disease risk in medicine.

1.5 Significance of this Study

Angiosperms flowers vary extensively, and this variation has stimulated scientific interest in plant sexual diversity and mating systems. Despite this attention, the evolution of this extraordinary diversity in ways of achieving "mating success" is still not fully understood (Barrett, 2002). Generally, mating systems studies have focused on the genetic causes and consequences (see Schemske and Lande, 1985; Charlesworth and Charlesworth, 1988; Holtsford and Ellstrand, 1990; Jarne and Charlesworth, 1993; Husband and Schemske, 1996). Nevertheless, a number of studies have also concentrated on the importance of ecological factors (such as availability of pollinators and mates) in shaping plant mating systems (Eckert and Schaeffer, 1998; Lu, 2000; Elle and Carney, 2003; Kalisz and Volger, 2003).

Today, studies of major reproductive transitions in flowering plants are the focus of considerable research in plant evolutionary biology (Barrett, 2008). Barrett (2008) has reviewed recent work by leading authors in the field of plant evolutionary biology with contributions featuring new research findings, reviews, and synthesis as well as including diverse approaches for understanding the pathways of reproductive-trait evolution in flowering plants. Also included in his review are comparative and phylogenetic methods, theoretical models, investigations of structure and development, molecular genetics, and experimental studies of the ecology and genetics of wild populations. However, he acknowledged that his review has not been comprehensive due to the exceptional reproductive diversity of flowering plants, and that significant major transition involving flower morphology and development, pollen biology, life history, as well as fruit and seed dispersal are for future consideration. Consequently, there are still questions unanswered in the

field of floral traits development, pollen and pollination biology as well as other life history traits.

Although there is a dearth of studies addressing the significance of floral morphological traits in shaping mating system evolution among species with variable mating systems, Mazer and Hultgard (1993) have reported that the results obtained from the measures of phenotypic and genetic variation and co-variation within and among floral traits of closely related species, that differ in mating systems can be used to provide answers to several evolutionary questions (see also Mazer and Delesalle, 2002). Therefore, the incorporation of information from quantitative genetics, floral development, and different functions of floral traits, within and between populations, in this study should provide important new insights into the roles of genetic constraint, floral developmental processes, and heritability (as well as evolvability) of these floral traits in the evolutionary process.

In addition, the data generated from this study will contribute to the ongoing research work (using *Collinsia* species as a model plant) to test the potential of different proposed measures of heritability including, evolvability (I_A) and additive genetic CV (see reviews in Lynch & Walsh 1998, Hansen *et al.*, 2003a, 2003b) as a method of providing insight into mating system evolution in plants. This concept, along with the evaluation of its likely measures, is not only central in quantitative genetics but also has important applications in evolutionary developmental biology (Armbruster *et al.*, 2002). Since there are also increasing societal needs for predicting the ability of organisms and traits to evolve, a practical heritability and evolvability measure will contribute extensively to forecasting the evolution of floral traits and mating systems in angiosperms, especially in plants with a mixed mating system.

1.5.1 Why *Collinsia heterophylla*?

Collinsia heterophylla has been chosen as a model plant for studying mating system evolution in plants, because it is easy to cultivate and the seeds germinate soon after sowing. Plants begin to flower at about ten

weeks after planting, and the flowering period lasts for about three weeks; thus, one life cycle takes about four months. The flowers are zygomorphic comprising five basally fused sepals, five basally fused petals, four stamens, one nectary and one pistil. The flower resembles a pea flower with keel, wings and banner. In *Collinsia heterophylla,* each ovary contains ca. 8 to 16 ovules and each stigma is capable of holding about fifty pollen grains at full pollen load (Armbruster *et al.,* 2002). The flowers are very easy to emasculate for cross-pollination treatments. Most *Collinsia* species exhibit a mixed mating system with delayed self-pollination that can allow high levels of out-crossing if pollinators are present and reproductive assurance if pollinators are absent (Charlesworth & Mayer, 1995; Kalisz *et al.*, 1999).

Species of *Collinsia* also show interesting within- and among-species variations in late floral development, which affects the timing of floral-part elongation and self pollination. Generally, the four anthers dehisce one at a time over 3-7 days; this has been numbered as stages 0 - 4 corresponding to the number of dehisced anthers. The staminal filament elongates just prior to anther dehiscence, placing the dehiscing anther at the tip of the keel petal. In most large-flowered species the style elongates late in development, whereas in most small-flowered species the style remains about the same length (Armbruster *et al.*, 2002). Self-pollination can occur when the stigma comes in contact with the anthers or with free pollen at the tip of the keel. However, dramatic variation in the stage of anther-stigma contact and stigma receptivity exists within and among species (Armbruster *et al.*, 2002) and this variation has a genetic basis (Lankinen et al. 2007). Hence *Collinsia heterophylla* could be used as a suitable model system for the study of mating system evolution in plants.

1.6 Investigation Outline and Aims

The following chapters give an account of research carried out as part of this investigation. The main aim of this study is to assess how phenotypic and genotypic variations in floral traits, as well as other interacting mechanisms, influence mating systems in plants, using two

populations of *Collinsia heterophylla* with different flower sizes and predicted mating systems. The small-flowered population is expected to be largely inbreeding, while the large-flowered population is expected to be largely outcrossing. To assess these mechanisms, floral morphological traits associated with mating systems are examined with the aim of understanding how these floral traits change with mating system evolution. The study addresses the following objectives:

(a) To investigate the correlations among the floral morphological traits measured and mating systems, as well as to estimate their heritabilities and discuss how these influence the mating systems in the two populations. This research is important because measures of phenotypic and genetic variation and co-variation within and among floral traits of closely related species that differ in mating systems can be used to generate and test several evolutionary hypotheses (see Mazer *et al.,* 2007).

(b) To assess the effect of four different pollination treatments (crossed - HC; selfed – HS; bagged – BG; and emasculated – EM) on flower longevity in the two populations of *C. heterophylla*, and to consider its implication for mating system evolution. This is because *C. heterophylla* is a self compatible plant, the timing of autonomous selfing could be important in determining its floral longevity, which could subsequently influence its evolution.

(c) To examine the pollen-tube growth in the two populations of *C. heterophylla, in-vitro* and *in-vivo,* and discuss how differences in pollen-tube growth rates can affect mating system evolution in this species. This is because pollen tubes have been reported to grow more rapidly in plants from an outcrossing population than in plants from a selfing population (see Kerwin and Smith-Huerta, 2000).

The first results chapter (Chapter 3) addresses Objective (a). Here I examine variations in eight floral morphological traits in two populations of *Collinsia heterophylla:* Sisar Creek, Southern California via Norway

(small-flowered) versus Northern California via Chiltern Seeds UK (large-flowered). Here variations in floral morphological traits within- and between-population are quantified and assessed for correlations and heritabilities. Correlations of the floral morphological traits with inferred mating system is then determined. In this chapter the following questions are addressed:

(i) Are there phenotypic and genetic variations in floral traits within- and between-populations?

(ii) Are floral characters associated with mating systems correlated within- and between-populations and do these traits correlate with mating systems?

(iii) What are the heritabilities and evolvabilities of these floral characters affecting mating systems? Would the effects of selection on these traits be passed on to the next generation as an evolutionary response?

(iv) Which of the floral traits will respond more to selection, or is selection stronger on other characters?

(v) Does anther-stigma contact differ significantly within- or between-populations?Also considered was the question: what is the average time of anther-stigma contact in each floral stage for each population?

The second results chapter (Chapter 4) addresses Objective (b). I investigate pollination-induced flower senescence and how fertilisation affects floral longevity in the two study populations of *C. heterophylla* and also the implications of pollination-induced flower senescence on mating systems evolution. According to Primack (1985), the optimal floral longevity depends on the breeding system of the species studied. However, Vogler and Kalisz (2001) opined that many species with animal-pollinated flowers are partially self-fertilising, meaning that these species of plants are more likely to undergo autogamy at some point during the life span of their flowers. Therefore, this chapter addresses the following questions:

(vi) Does the timing of fertilisation affect the rate of flower senescence?

(vii) Does type of pollination (self or outcross) affect the rate of flower senescence?

(viii) Does flower longevity differ between these two populations under the same pollination treatments and environmental factors?

(ix) Does emasculation increase floral longevity?

The final results chapter (Chapter 5) addresses Objective (c). Here I examine pollen-tube growth *in-vivo* and *in-vitro* for the two study populations of *Collinsia heterophylla,* the two populations are compared to assess the pattern of pollen-tube growth and describe how this differs with inferred mating system. Pollen tubes have been reported to grow more rapidly in plants from an outcrossing population of *Clarkia tembloriensis* than in plants from a selfing population (Kerwin and Smith-Huerta, 2000). However, Lankinen et al., (2009), have examined pollen-tube growth rate in the southern California population of *C. heterophylla,* and reported that there is no significant difference in pollen-tube growth rate *in-vitro* and *in-vivo* within this population (see also Skogsmyr and Lankinen, 2002). This chapter therefore sets out to assess pollen-tube growth rates in-*vitro* and *in-vivo* in two populations of *C. heterophylla* in order to examine how pollen performance differs between small-flowered (Southern California via Norway) and large-flowered (Northern California via Chiltern) populations; and how this difference in turn could influence the evolution of the competitive ability of the male gametophyte with the consequence of affecting mating system evolution in this species. The following questions are asked:

(x) Do *in-vitro* and *in-vivo* pollen-tube growth rates differ within and between the two populations of *C. heterophylla* studied*?*

(xi) Is there any difference in pollen-tube growth on self versus cross styles for the two populations of *C. heterophylla* studied?

(xii) What are the implications of different pollen-tube growth rates for mating systems evolution?

Chapter 6 concludes with a general discussion of the evolution of mating systems in plants and how it is being influenced by floral characteristics, as well as the evolutionary implications of the correlations and heritability of floral morphological traits on mating systems evolution in *C. heterophylla*. In addition, the major findings are summarised and areas of proposed further research work are highlighted.

CHAPTER 2

2.0 Study Species and Experimental Procedures

2.1 Study Species

The study species is a member of the genus *Collinsia.* Previous research has revealed significant morphological and mating-system variations within and among species in this genus, allowing comparison of morphology, development, mating systems, and the correlation, as well as inheritance of these traits.

2.1.1 Background on the Genus *Collinsia*

The genus *Collinsia* (Scrophulariaceae .s. lat., Plantaginaceae sensu Judd *et al.*, 2002) is monophyletic (Randle et al., 2009) and consists of approximately 20 species restricted to North America. Most species occur in California, although many are found in other parts of Western North America and three species are found east of the Mississippi (Schrock and Palser, 1967). All species are annuals; seeds germinate and plants grow vegetatively in the winter or early spring and bloom in the early spring to early summer.

2.1.2 Biology of *Collinsia heterophylla*

Collinsia heterophylla is a hardy annual that is endemic to California and North-western Mexico. They grow to between 30 – 50 cm tall and spread 15 – 20 cm, bearing whorls of pretty flowers ranging in colour from white through lilac and rose to violet and blue. They flower somewhat before the end of spring (March through June), depending on latitude, elevation, and shading. The leaves of *C. heterophylla* are lanceolate, scalloped edges, and with cordate bases.

Figure 2.1 Picture of *Collinsia heterophylla* growing in pots outside the greenhouse of the University of Portsmouth, UK in spring/summer of 2007.

2.1.3 Inflorescence

The common name “Chinese houses” comes from the arrangement of the inflorescence. Numerous flowers are arranged in a tiered or whorled spike similar to tiered Chinese pagodas.

2.1.4 Flower Morphology

The flowers of *Collinsia heterophylla* are pedicellate and zygomorphic, i.e. only one plane of symmetry. Floral parts comprise five basally fused sepals (calyx), five basally fused petals (corolla), four stamens, and one bi-carpellate pistil. The petals are normally arranged in an upper lip of two lobes called the banner and a lower lip of three lobes including a pair of wings with a folded (conduplicate) keel, enveloping the style and stamens. At the base of the banner, wings and keel, the corolla is constricted into a narrow opening, thus forming a constricted ‘mouth’ at the top of a saccate tube (Figure. 2.2). The four stamens are borne on the

corolla (epipetalous), as is the small basal nectary, which is probably of staminal origin. The pistil comprises a style of variable length, a small, terminal, bi-lobed stigma, and an ovary enclosing 2-16 ovules (Armbruster *et al.*, 2002). All *Collinsia* pistils are bi-carpellate Gorsic, (2003). However, there are occasional spots of free carpels.

2.1.5 Corolla

The corolla colour is normally dark purple on the lower lip and white to pale purple on the upper lip. Some populations show some dark purple on parts of the upper lip while others show pale purple or off-white on both lips (pers. obs.).

Figure 2.2: Picture of *Collinsia heterophylla* Norway population showing the whorled arrangement of the flowers/inflorescence on the axis – Chinese pagoda.

2.1.6 Stamens

The four stamens have been described as four didynamous stamens that is, they are borne in two pairs, the upper pair and the lower pair. Members of the same staminal pair are of the same length for much of the life of the flower and they dehisce at similar but not identical times. The stamens and the style elongate with age, the elongation of the stamen stops when the anther dehisces (pers. obs).

2.1.7 Anthers

The anthers usually dehisce one at a time over a period of 3-4, days (occasionally 2-5 days) depending on the prevailing environmental conditions during flowering (Armbruster *et al.*, 2002; Gorsic, 2003). The anthers dehisce starting from one of the lower stamens and then the other and after that one of the upper and finally the second upper stamen. In some plants it has been observed that the stamens do not dehisce but seem to die off early during flower development (pers. obs); consequently, no fruits are produced by such flowers except when the stigma of such a flower receives pollen grains from other plants of the same species. The reason for this developmental abnormality could not be ascertained as *Collinsia* is not known to undergo male sterility. This condition was, however, found to be prevalent when *C. heterophylla* plants were exposed to environmental stress.

2.1.8 Style

In most species of *Collinsia*, the style elongates during the period of flower development; the stigma becomes receptive and is eventually brought in contact with the dehisced anthers. This consequently allows self pollination to occur (Kalisz *et al.*, 1999; Armbruster *et al.*, 2002), and the ovaries develop into dry, dehiscent seed capsules. However, personal observation shows that, when environmental conditions are harsh, some small-flowered plants produce flowers with styles elongating faster than the stamens and protruding through the two-lobed wings, while the stamens remain enclosed in the keel.

2.2 Experimental Procedures

2.2.1 Sampling

The seeds of *Collinsia heterophylla* used for this research were collected from two different sources. One set of seeds were obtained from the Norwegian University of Science and Technology, Trondheim, Norway; these seeds were originally collected from a pale-purple flowered *C. heterophylla* in Sisar Canyon, Ventura County, Southern California (see Population 4 in Armbruster *et al.,* 2002). The seeds were transported from California to Norway and were grown in the greenhouse of the Norwegian University of Science and Technology, Trondheim, where inbred lines were developed; and seeds from these inbred lines were brought into England by W. S. Armbruster in 2004. This population is a line of small-flowered *Collinsia heterophylla* generally referred to as the Norway population throughout this thesis. The second set of seeds was obtained from Chiltern seeds, Bortree Stile, Ulverston, Cumbria, England, UK. This population of *Collinsia heterophylla* is thought to have been brought into Britain from Northern California in the 19th century. This population is a line of large-flowered *Collinsia heterophylla* growing freely in the fields at Chiltern seeds garden in the UK; the plants were allowed to set seeds through open pollination. These two populations were used because they showed a distinct variation in flower size. The Norway population produced smaller flowers, on average, while the Chiltern population produced larger flowers. In the Norway population flowers were generally pale purple, while the flowers in the Chiltern population were mostly dark purple.

The Norway population was selfed during spring/summer of 2005 in the green house of the University of Portsmouth, UK. The seeds collected from this 2005 population formed the pre-parental generation for Norway population because the seeds were too few to make a parental generation. The Chiltern population obtained from Chiltern seeds arrived in 2006. These seeds were grown in the greenhouse of the University of Portsmouth, UK, and plants were selfed to produce a pre-parental

generation. Plants were selected for large flowers and their seeds harvested and replanted to generate the parental generation for the Chiltern population.

2.2.2 Experimental Site

The plants were grown in the newly constructed greenhouse of the University of Portsmouth, behind Anglesea building. The seeds of *C. heterophylla* were left to grow in the greenhouse each year throughout the experimental period. Seeds were grown under natural sunlight during the growing seasons of 2006, 2007, 2008 and 2009. During the growing period, temperatures were maintained at 25°C to 30°C during the day and 16°C to 22°C during the night.

Figure 2.3 Picture of *Collinsia heterophylla* Norway population in the Greenhouse University of Portsmouth in Spring/summer 2007

2.2.3 Seed Propagation

Collinsia heterophylla seeds were sown in nursery trays filled with multipurpose compost and placed in the greenhouse during late January and early February each year, because the seed germinated better at this period of the year. Most of the seeds (60% – 80%) started to germinate at about 14 days after planting. Throughout the experiment, germination rate greater than 95% was never recorded. However, germination rate varied between the two populations of *C. heterophylla* used, with the Norway population measuring between 50 – 65% approximately, while the Chiltern population had a score of between 80 – 95% approximately.

The seedlings were generally transplanted two to three weeks after planting, when the second pair of leaves had emerged. Single seedlings were transplanted into separate plastic flower pots filled with multipurpose compost. The number of plants raised each year varied as the conditions in the greenhouse was very inconsistent and unpredictable, this was largely due to the fact that *Collinsia heterophylla* proved to be very sensitive to high temperatures, strong sunlight, as well as susceptible to pests such as white and green flies. It was therefore not possible to raise very large populations of *Collinsia heterophylla* in the greenhouse for the purpose of the experiments presented in this thesis. Even when the germination rate was very high (for example 80 per cent), only about half of the population survived to flowering.

2.2.4 Experimental Design

The experimental design used for the experiments in this thesis is the randomized complete block (RCB) design. Flower pots were placed in a randomized array and for most of the vegetative growth phase, the entire array was shifted every three days in a systematic way to reduce the effects of the micro-environmental variation.

2.2.5 Data Collection and Analyses

2.2.5.1 Variability, Correlations and Heritabilities of Floral Traits Within- and Among-Population of *Collinsia heterophylla*

The first objective of this research is to measure and assess the variability, correlations and heritability of floral traits influencing mating systems in *Collinsia heterophylla.* The eight floral morphological traits measured (as shown in Fig. 2.4) are: corolla length (from base of sepals to opening of corolla tube), corolla/wing width (horizontal inside diameter), gynoecium/pistil length (from base of sepals to tip of stigma), androecium/stamen length (from base of corolla to the base of the anther for stamens at stage1 and stage 3), keel length (from base of corolla tube to tip of keel), banner length, and anther-stigma contact (ASC; i.e. the point during floral development when the stigma comes in contact with the dehiscing anther). Data is collected and analysed as described in chapter 3 (see section 3.2 for details).

2.2.5.2 Flower Development and Longevity

The second objective of this research is to study how four pollination treatments affect flower longevity in the two populations of *Collinsia heterophylla* and its implication for mating system evolution. The pollination treatments applied to the two populations are as follow: (i) crossed (HC); (ii) bagged (BG); (iii) selfed (HS); and (iv) emasculated (EM). A fifth group is added to the pollination treatments: unmanipulated (UM) – these are open-pollinated flowers (i.e. not bagged and no treatment applied) - serving as the control. The pollination treatments are compared to test the hypothesis that fertilisation triggers flower senescence (see section 4.2 in chapter four for details).

2.2.5.3 Pollen-Tube Growth Rate

The third objective of this research is to examine the *in-vitro* and *in-vivo* pollen-tube growth rates in the two populations of *C. heterophylla* studied, and to assess how the differences in pollen-tube growth rates can affect mating-system evolution in *C. heterophylla.* In chapter 5 pollen-tube

growths are measured in a growth medium (Hoekstra medium) and on the styles of recipient plants in the two populations of *C. heterophylla* studied (see details in section 5.2).

CHAPTER 3

3.0 Within- and Among-Population Variability, Correlations and Heritabilities of Floral Traits in *Collinsia heterophylla*

3.1 Introduction

3.1.1 Mating Systems

The diversity of mating systems in plants has fascinated biologists since the time of Darwin's monographs in 1876 and 1877 (Barrett 2003). Mating systems are significant determinants of population genetic structure and evolutionary potential. The evolution of mating systems is determined by both genetic and ecological factors (Figueroa-Castro, 2008), some of the characteristics that affect mating systems (e.g. floral morphology and developmental timing) are quantitative traits. These traits have been observed to influence the distribution of genetic diversity within and between populations (Charlesworth and Charlesworth, 1987 and 1995; Hamrick and Godt, 1996; Charlesworth and Wright, 2001; Holeski and Kelly, 2006). Therefore, changes in these traits are often closely linked with shifts between outcross and self pollination, which will consequently cause changes in plant mating systems (Jain, 1976; Steven and Waller, 2004; Van Kleunen and Ritland, 2004; Figueroa-Castro, 2008).

Outcrossing plants usually have a high production of large showy flowers with relatively long flowering times. They may be protandrous, protogynous, and/or herkogamous, with anthers and stigmas spatially separated and high production of pollen grains compared to ovules. In comparison, selfing species generally have smaller, less showy flowers, shorter flower longevity, less temporal and spatial separation of male and female functions and lower pollen: ovule ratios (Cruden, 1977; Morgan and Barrett, 1989; Dole, 1992; Parker, *et al.*, 1995; Karron *et al.*, 1997; Chang and Rausher, 1998; Sun, 1999; Fishman, *et al.*, 2002; Suso, *et al.*, 2003; Steven and Waller, 2004; Van Kleunen and Ritland, 2004; reviewed in Figueroa-Castro, 2008).

Although, several models of the evolution of plant mating systems predict that plants should evolve towards either complete self-fertilisation or complete outcrossing (Lande and Schemske, 1985; Charlesworth *et al.*, 1990; Uyenoyama *et al.*, 1993; Lande *et al.*, 1994; reviewed in Davis and Delph, 2005); studies investigating selfing rates in natural systems have pointed out that mixed-mating systems (i.e. those that combine both selfing and outcrossing) may be more common than have been predicted by models of mating systems (Brown and Clegg, 1984; Barrett, *et al.*, 1996; Vogler and Kalisz, 2001). Mixed mating systems can also occur in species that produce two types of flowers on the same plant. For example, some plants produce both cleistogamous (obligate selfing) and chasmogamous (outcrossing) flowers, (e.g., *Impatiens capensis*; Schemske, 1978; Lu, 2000). In contrast other plants (e.g. *Collinsia heterophylla*) produce flowers that are capable of both selfing and outcrossing depending on the pollinator availability (Armbruster *et al.*, 2002). According to Takebayashi (2000), among-plant variation in herkogamy may lead to variation in the amount of selfing among plants within populations. The selfing that does take place as a result of such variation in floral traits may occur at different times within an individual flower's life span. Different modes of autonomous self pollination, focus on the timing of selfing relative to outcrossing (i.e. prior, competing, and delayed; Lloyd 1992), which occur within flowers, can also result in a mixed mating system (Davis and Delph, 2005; Fenster and Martén-Rodríguez, 2007). However, the ecological conditions under which each mode may be selected for differ. Selection for reproductive assurance will favour prior selfing (i.e. selfing before any opportunity for outcrossing) only if the chance of being outcrossed later is very low (persistent pollinator limitation) and/or inbreeding depression are weak (Lloyd, 1992; Elle and Hare, 2002). In contrast, delayed selfing (i.e. selfing after all opportunities for outcrossing are past), is favoured by selection almost all the time, because it provides reproductive assurance without limiting opportunities for outcrossing via either seeds or pollen (Lloyd, 1992; Davis and Delph, 2005).

Most studies have focused on the evolution of mating systems and on how the balance of evolutionary advantages and costs determine the tendency for plants to self-fertilise versus outcross (see Uyenoyama *et al.*, 1993). In addition, models and empirical studies attempting to explain the maintenance of mixed mating within populations have mainly focused on the effects of inbreeding depression (Uyenoyama and Waller, 1991; Rausher and Chang, 1999; Cheptou and Schoen, 2003), seed and pollen discounting (Holsinger 1988; Herlihy and Eckert 2002), and reproductive assurance (Lloyd, 1992; Kephart *et al.*, 1999; Kalisz and Vogler, 2003; Tsitrone *et al.*, 2003; reviewed in Holeski and Kelly, 2006; see also Davis and Delph, 2005). However, according to Holeski and Kelly (2006), there is an important question that has received less empirical study, this question is, 'how do differences in mating systems impact the evolution of quantitative traits' (Holeski and Kelly, 2006)?

3.1.2 Floral-Traits Evolution

Angiosperms exhibit tremendous variation in floral traits such as the size and shape of the corolla, the quantity and quality of rewards offered to pollinators, and the positions of sexual organs. This variety is thought to have evolved primarily as a result of natural selection generated by animal pollinators (e.g. Baker and Hurd, 1968; Stebbins, 1970; reviewed in Campbell, 1996). These floral reproductive and developmental traits are all quantitative traits (reviewed in Holeski and Kelly, 2006).

3.1.3 Floral- and Reproductive-Traits Variations

The size and number of flowers displayed together on an inflorescence (floral display) influence pollinator attraction as well as pollen transfer and receipt is this is fundamental to plant reproductive success and fitness (Sargent, *et al.*, 2007). Plant reproductive success and pollinator behaviour are regularly influenced by floral and reproductive traits and such traits are therefore expected to be under selection (Mitchell and Shaw, 1993; Mitchell, 2004). According to Sargent *et al.* (2007), two important aspects of floral display (i.e. flower size and flower number) have

definite effects on pollinator visitation rate and total seed production (see Bell, 1985). As a result, it could be predicted that selection for increased pollinator visitation rate should increase both the flower size and the flower number (Sargent *et al.*, 2007). However, there is little information on the amount of genetic variation in such traits in wild plant populations (Mitchell and Shaw, 1993; see also Ashman and Majestic, 2006; Caruso, 2006). Although, according to Williams and Conner (2001), phenotypic variation in floral morphology has shown susceptibility to pollinator-mediated natural selection, and this variation has been shown to be heritable, but not much information is available concerning the sources of floral variation. Therefore, in order to gain full understanding of floral trait evolution, it is essential to establish the sources of variation in floral morphology.

Primack (1987) reported that large plants have a tendency to produce larger flowers than small plants; hence, any factor that affects the vegetative morphology of a plant is likely to influence the flower size (see also Armbruster et al., 1999; Caruso, 2000; Caruso, 2006). Such associations can influence the heritability of floral morphology and other traits expressed late in the ontogeny. Therefore, if vegetative size is determined by growth conditions, environmental heterogeneity is expected to influence the expression of heritable floral variation, thereby slowing the evolutionary response to selection (Andersson, 1996; Cresswell *et al.*, 2001; Ashman and Majestic, 2006). In addition, if plant size has a strong heritable component, then floral traits would automatically show genetic variation, leading to high heritabilities for these characters (see Gómez *et al.,* 2009). Thus, quantitative genetic analyses of data adjusted for overall plant size will provide an opportunity to decide whether genetic variation in floral traits is caused by genes expressed during flower development or genes influencing plant size (Robertson *et al.*, 1994; Meagher, 2007).

Furthermore, it has been reported that spatial and temporal variation can have diverse effects on selection on floral traits; for example, Conner and Via (1993) observed that heritabilities of wild radish floral traits were much lower in the field than in a very similar greenhouse

experiment and that this was due in part to increased environmental variation in the field. However, in 2001 Williams and Conner studied phenotypic variations in seven floral traits in wild radish (*Raphanus raphanistrum*) and observed that there were no significant differences between plants derived from the two source populations, which were approximately 800 km apart. Most of the phenotypic variance they observed was within individual plants. Consequently, Williams and Conner (2001) proposed that variation within individual plants could be divided into "spatial" variation (i.e. variation among flowers open at the same time on a plant), and "temporal" variation (i.e. variation among flowers open at different times across the flowering period of each individual). In addition, it was suggested that temporal variation in floral traits could be the result of ontogenetic changes as well as alteration in the environment over time. For instance, if there are temporal changes in floral size, then pollinators on each given day will tend to select the same plants and selection will not be weakened. However, different individual plants maintain their relative status over time (i.e., no time-by-individual interaction). In contrast, if flower size varies more within individuals than among them, or there is a strong time-by-individual interaction, then pollinators will not repeatedly choose one plant and selection will be weakened (Williams and Conner, 2001).

Nevertheless, this pollinator-mediated selection is influenced by the phenotypic variation among individuals in a population. This means that if there is more variation within an individual than among individuals, selection and the evolutionary response to selection will be weakened, because individual plants in many species produce several to many flowers (e.g., Darwin, 1877; Campbell, 1989; Galen, 1989; Schemske and Horvitz, 1989; Williams and Conner, 2001). For example, if each plant produces flowers with a broad variety of floral characteristics, pollinators are less able to select among the flowers of different plants. However, if different plants consistently produce different flowers, pollinators may go to one plant more often than another and as a result will select for specific

floral traits (Williams and Conner, 2001). Therefore, variation in floral traits can influence the evolution of mating systems in plants.

3.1.4 Floral-Traits Correlations

Floral development is highly integrated, and this can lead to ontogenetic correlations between traits associated with pollinator attraction and reward (Armbruster, 1991). Genetic correlations among traits are important in evolution, as they can constrain evolutionary change or reflect past selection for combinations of traits (Lande and Arnold, 1983; Maynard Smith *et al.*, 1985; Mitchell-Olds and Rutledge, 1986; Deng *et al.*, 1999, reviewed in Conner, 2002). Genetic correlations in an organism may be caused by pleiotropic and developmental relationships among traits and may produce constraints on evolution by natural selection (reviewed in Conner and Via, 1993). On the other hand, selection may directly change the patterns of genetic correlations particularly, in cases where two or more traits interact to carry out a given function (Cheverud, 1984; Lande, 1984; Clark, 1987a & b, reviewed in Conner and Via, 1993). But the genetic mechanisms underlying correlations remain largely unknown in natural populations (Clark, 1987a & b; reviewed in Conner, 2002). However, Conner (2002) provides direct evidence of the genetic mechanisms underlying correlations between quantitative traits. He reported that after nine generations of random mating (nine episodes of recombination) correlations between six floral traits in wild radish plants were unchanged, showing that pleiotropy generates the correlations. No evidence for linkage disequilibrium was observed despite previous correlational selection acting on one pair of functionally integrated traits (Morgan and Conner, 2001).

It has also been reported that positive correlations among traits can be caused by larger genetic variation in resource acquisition than in allocation among individuals (see Houle, 1991; Davis, 2001; Vorburger, 2005); consequently, individuals with additional resources available would distribute more to both male and female traits. Besides, patterns of correlations among floral and vegetative morphological traits may mirror

past selection on these traits. This is caused by differential pollination success, given that selection can theoretically increase or decrease correlations (Conner and Via, 1993; see Davis, 2001), and genetic correlations between two traits that interact to perform a given function can also be shaped by selection. However, if functional integration between traits increases an individual's fitness, selection would act to increase the positive correlation between those traits (Conner, 1997; Conner and Via, 1993; reviewed in Davis, 2001). Hence, positive phenotypic and genotypic correlations between parts of the same reproductive organ (e.g. the anther and filament of the stamen) indicate developmental association between these traits and as a result caused by pleiotropy or linkage (Davis, 2001; Conner, 2002).

3.1.5 Floral-Traits Heritability

The heritability (h^2) of a trait, that is, the proportion of the total phenotypic variation that is explained by additive genetic variance (V_A), is the extent to which phenotypic selection on parents will influence the distribution of that trait in progeny genotypes. Phenotypic selection operates frequently in plant populations and at multiple stages in the life cycle (Young *et al.*, 1994). Falconer (1989) has shown how traits with low heritability respond more slowly to selection than traits with higher heritability. Therefore heritability estimates of various traits are helpful in predicting which traits will respond most rapidly to selection (Falconer and Mackay, 1996).

Traits that are closely associated with fitness have been generally considered to have low heritabilities, because stabilizing selection on these traits over time has acted to reduce the genetic variation (Stearns, 1980; Falconer, 1989; Merilä and Sheldon, 2000; McCleery, *et. al.,* 2004). However, several studies have reported fairly high heritabilities of traits closely correlated with fitness (Giesel *et al.*, 1982; Mitchell-Olds, 1986; Mousseau & Roff, 1987; see detailed review in Young *et al.*, 1994; Sinervo and Zamudio, 2001). Similarly, heritabilities of ecologically important traits have been reported to be generally moderately high in a variety of

organisms (e.g. Roff, 1997; Merila & Sheldon, 2000; reviewed in Keller *et al.*, 2001); this is evolutionarily significant because, it implies that the traits are responsive to selection. However, the concept of measuring the standardised variation of V_A (i.e. 'evolvability'; Houle, 1992) for different traits is helpful because of the inherent relationship between the amount of genetic variation of a trait and the rate of response to selection. Thus if a population has little genetic variation for a trait, the response to selection will be very slow, even if the trait exhibits strong heritability (Young *et al.*, 1994); this is in fact unlikely as the reverse is the case. Conversely, a trait could have high evolvability but low heritability (W.S. Armbruster, pers. com. 2009).

Floral-trait heritability has been observed to be fairly significant among plant species; for example, Mitchell and Shaw (1993) experimented with the perennial wildflower *Penstemon centranthifolius* and observed that most traits showed significant heritability, including nectar production, corolla length and width, inflorescence length and total flower production. Young *et al.* (1994) also measured heritability and genetic correlations of three floral traits (corolla width, pollen production per flower and pollen size) in two generations of wild radish (*Raphanus sativus*) grown in three growth environments (two field sites and the greenhouse). They found that corolla width and pollen production showed significant heritabilities in both generations and under all growth environments. In addition, Charlesworth and Mayer (1995) measured quantitative genetic variability for six characters in four populations of *Collinsia heterophylla.* They observed that all four populations showed significant genetic variation for two or more characters and did not record any lower amount of genetic variability or lower heritability in the more selfing populations. Furthermore, Ashman and Majestic (2006) reviewed data on heritability and genetic correlations for several classes of floral traits (primary sexual, attraction and mating system) in hermaphroditic plants and found significant heritability and also variations among all floral traits; but not life history. They also found that there is a tendency for heritability to vary with mating system.

3.1.6 Reasons for the Study

Although phylogenetic comparison among species is a potent tool for the study of evolution (Harvey and Pagel, 1991; Armbruster, 1992; Weller and Sakai, 1999), detailed studies of variation within populations are also required to reach a deeper understanding of evolutionary mechanisms (Lankinen *et al.*, 2007). For instance, in order to predict how traits would respond to selection, the estimate of heritable variation of such trait is critical. There is only limited information of such variation in floral developmental traits, such as flower size, size of flower parts (for example lengths of sepals, petals, stamens and pistils) or timing of stigma receptivity and anther-stigma contact (but see Charlesworth and Mayer, 1995; Armbruster, 2002 and Lankinen *et al.*, 2007).

In addition, Williams and Conner (2001) reported that a number of studies have measured pollinator-mediated selection and the heritability of floral traits (Galen, 1989; Johnston, 1991; Conner and Via, 1993; Carr and Fenster, 1994; Campbell, 1996; Conner *et al.*, 1996), and others have examined variation in floral traits among populations and among plants (Waser and Price, 1984; Schwaegerle *et al.*, 1986; Herrera, 1990); however, only a few studies have measured variation among flowers within plants (e.g., Campbell, 1992; Svensson, 1992; Dominguez *et al.*, 1998). Besides, in *Collinsia heterophylla*, an ideal model plant for studying mating systems in plants, there are very few empirical data on the heritability and evolvability of floral traits (but see Lankinen *et al.*, 2007). Lankinen *et al* (2007) experimented on the Southern California (mostly small-flowered) population of *C. heterophylla* and used father-offspring regression to estimate heritability of the floral traits studied. The current research thesis investigates both Northern and Southern California populations (i.e. predominantly large-flowered and mostly small-flowered, respectively), and use parent-offspring regressions (i.e. mid-parent – offspring regression) to estimate heritabilities of the eight floral traits measured. The results obtained from this experiment will broaden our understanding of the correlations, heritabilities and evolvabilities of the floral traits studied. Also, the knowledge of the heritability and evolvability

as well as correlations among these floral traits will enable us to better understand the evolution of mating systems in *Collinsia heterophylla*.

Therefore, the aim of the research in this chapter is to investigate the phenotypic and genetic correlations among the eight floral morphological traits mentioned in 2.3.1.1 above; and how these may affect the evolution of mating systems in *C. heterophylla*. This study intends to use the mid-parent-offspring regression to estimate the heritability of the eight floral morphological traits, measured in the two populations of *C. heterophylla* grown under the greenhouse conditions, because the average of the two parents will give us an estimate of the total narrow-sense heritability. Young, *et al.* (1994) found heritabilities of floral characteristics in wild radish to be highly significant, even under greenhouse conditions. In this chapter, the following research questions were addressed:

1. Are there phenotypic and genetic variations in floral traits within- and between-populations?
2. Are floral characters associated with mating systems correlated within- and between-populations and do these traits correlate with mating systems?
3. What are the heritabilities and evolvabilities of these floral characters affecting mating systems? Would the effects of selection on these traits be passed on to the next generation as an evolutionary response?
4. Which of the floral traits will respond more to selection, or is selection stronger on other characters?
5. Does anther-stigma contact differ significantly within- or between-populations? Also considered was: what is the average time of anther-stigma contact in each floral stage for each population?

3.2 Materials and Methods

3.2.1 Study Species

Collinsia heterophylla is a diploid ($2n$ = 14) and self-compatible annual native to California Floristic Province with a mixed mating system (Newsom, 1929; Neese, 1993; Armbruster *et al.*, 2002). Plants are widely distributed and grow in shady places and dry slopes (<1000 m). They flower between March and June depending on latitude and elevation. The flowers are zygomorphic comprising five basally fused sepals, five basally fused petals, four epipetalous stamens, one nectary and one pistil. They are arranged in terminal whorls in spike-like inflorescences and pollinated by a variety of native bees (Armbruster *et al.*, 2002). The flowers resemble the pea flower with two upper banner petals, two lower wing petals, and the sexual parts are contained within a folded keel petal. The petals unite at the mouth of the flower to form a corolla tube, which is mildly to strongly saccate on the upper side, forming a pronounced bend. The small nectary is located on the upper side of the base of the ovary, near the saccate bend in the corolla (Elle, 2004).

During the course of development of the flower in *C. heterophylla*, the four anthers dehisce one at a time (that is, approximately over a period of four days); the first anther usually dehisces one day after anthesis. Throughout this period, the style elongates and the stigma becomes receptive; this eventually places the stigma in contact with the dehisced anthers, and self-pollination can occur (Kalisz *et al.* 1999; Armbruster *et al.* 2002). However, delayed self-pollination may occur as a result of the distal position of the stigma to the anthers from the earliest stages; even though it has been observed that delayed self-pollination is as efficient as early self-pollination. In addition, the work of Armbruster *et al.* (2002) shows that autonomously self-pollinated flowers set as many seeds as emasculated hand self-pollinated flowers. Equally, when self-pollination is delayed and pollinators are present, high levels of out-crossing is allowed and hence reproductive assurance. This is because *Collinsia* species generally exhibit a mixed mating system (Charlesworth and Mayer,

1995; Kalisz *et al*, 1999). Estimates of mean population outcrossing rates, ranging from 0.32 to 0.64, based on allozyme markers, have been reported by Charlesworth and Mayer (1995); while Weil and Allard (1964) reported up to 0.94 ± 0.27 based on morphological markers. Flowers of *C. heterophylla* develop into dry, dehiscent capsules, each containing up to 16 seeds (Armbruster *et al.*, 2002).

Although the developmental characters (e.g. floral traits, timing of pollen stigma contact and stigma receptivity) important for mating systems in *Collinsia* species have a quantitative genetic basis, they also vary continuously among and within populations (Armbruster *et al.*, 2002; Elle, 2004). The two populations of *C. heterophylla* used for this study have been categorized into the Chiltern population (generally large-flowered) and the Norway population (majorly small-flowered). The two populations originated from two different locations in California. One population originated from Southern California and was grown in the Norwegian University of Science and Technology greenhouse between 2002 and 2003. Seeds obtained from this population were brought into the UK in 2004 for the purpose of this research. This population is referred to as the Norway population. The second population originated from Northern California and cultivated in the field at Chiltern Seeds in UK. Seeds from this open-pollinated population were obtained and selfed in the University of Portsmouth greenhouse. This population is known as the Chiltern population for the purpose of this study. These two populations were selected because they vary distinctly in flower size. The Norway population is largely small-flowered, while the Chiltern population is mainly large-flowered. This will make it possible to study floral traits variations as well as the correlations in these floral traits within and between populations. The results obtained will provide insight into how floral traits correlate with mating systems and thus influence the evolution of mating systems in plants.

3.2.2 Experimental Site and Propagation

The plants were grown in the newly constructed greenhouse of the University of Portsmouth, behind Anglesey building. Plants were left to grow under natural sunlight during the summer of, 2007, 2008 and 2009. It was not possible to grow these plants during the winter months because of the conditions in the greenhouse; and every effort to create an artificial growing condition with the required temperature and sunlight proved unsuccessful. During the growing period, temperatures were maintained at 25^{o}C to 30^{o}C during the day and 16^{o}C to 22^{o}C during the night. Seeds of *Collinsia heterophylla* were sown in the greenhouse during late January/early February each year. *C. heterophylla* seeds from the two populations were broadcast on multipurpose compost in nursery trays and then covered with a thin layer of compost. To obtain maximum germination, the nursery trays were covered with a nursery tray lid for the first two weeks (until seeds started to germinate) in order to create the optimum humidity for the seeds to germinate (see details of C. heterophylla seeds propagation in section 2.2.3).

3.2.3 Experimental Design and Data Collection

Collinsia heterophylla varies continuously in flower size at the different flower developmental stages and this is considered to be of great value for the examination of the relationships between variations in flower size and correlation in floral traits (see Armbruster *et al.*, 2002). A randomized complete block experimental design was used. To assess genetic variability in flower size, floral traits were measured (on the two populations studied) during the flowering period of 2007, 2008 and 2009. On each plant five flowers were measured at each of the five floral developmental stages (i.e. 0, 1, 2, 3 and4), from day one of anthesis to day four/five. Each measurement was repeated three times to increase accuracy and precision. Hence 5 flowers x 5 floral stages x 3 repeated measurements = 75 measurements x 8 floral traits were made per plant. Flowers on an individual plant did not all open on the same day and so, in most cases, it was not possible to take all measurements on a single plant

in one day. In addition, due to variation in the time of flowering, flowers were measured over a period of 3 – 6 weeks each year.

3.2.3.1 Flower Collection and Measurements

Selection of plants to be measured was done at the time of flowering and measurements were made on properly formed flowers. Flowers from individual plants were collected and measured directly in the greenhouse throughout the period of the research. However, some flowers were fixed and stored in 70% ethanol for the purpose of measurement in the laboratory, following the procedure described by Kalisz *et al.*, (1999); and Armbruster *et al.*, (2002). Flowers were classified into five stages based on the number of dehisced anthers (i.e. zero – no dehisced anther to four – all four anthers dehisced) as describe by Armbruster *et al.* (2002). Flower samples in the greenhouse were examined with a magnifying glass (i.e. hand lens) and measured with digital callipers, while flowers stored in 70% ethanol were examined under a dissecting microscope and measured with an ocular micrometer.

The eight floral morphological traits measured are as shown in Figure 3.1 : corolla length (from base of sepals to opening of corolla tube), corolla/wing width (horizontal inside diameter), gynoecium length (from base of sepals to tip of stigma), androecium length (from base of corolla to the base of the anther for stamens at stage1 and stage 3), keel length (from base of corolla tube to tip of keel), banner length, and anther-stigma contact (ASC) [i.e. the point during floral morphological development when the stigma comes in contact with the dehiscing anther]. Other traits measured include; stigma receptivity, ovule number, plant height (at flowering and at senescence), number of branches at maturity, number of flowers, number of fruits/plant and average number of seeds/fruit. However, some of these traits measured were not used in the analysis. All floral morphological traits were measured in millimeters (mm) to the nearest 0.01mm with Mitutoyo Absolute Digimatic digital calipers and measurements were entered into the computer using an input device. The input device was used in order to ease the process of transferring data

from the log book into the computer and also to reduce the error of transferring handwritten data into the computer. Other vegetative morphological traits (e.g. plant height) were measured with a measuring tape to the nearest 0.01cm.

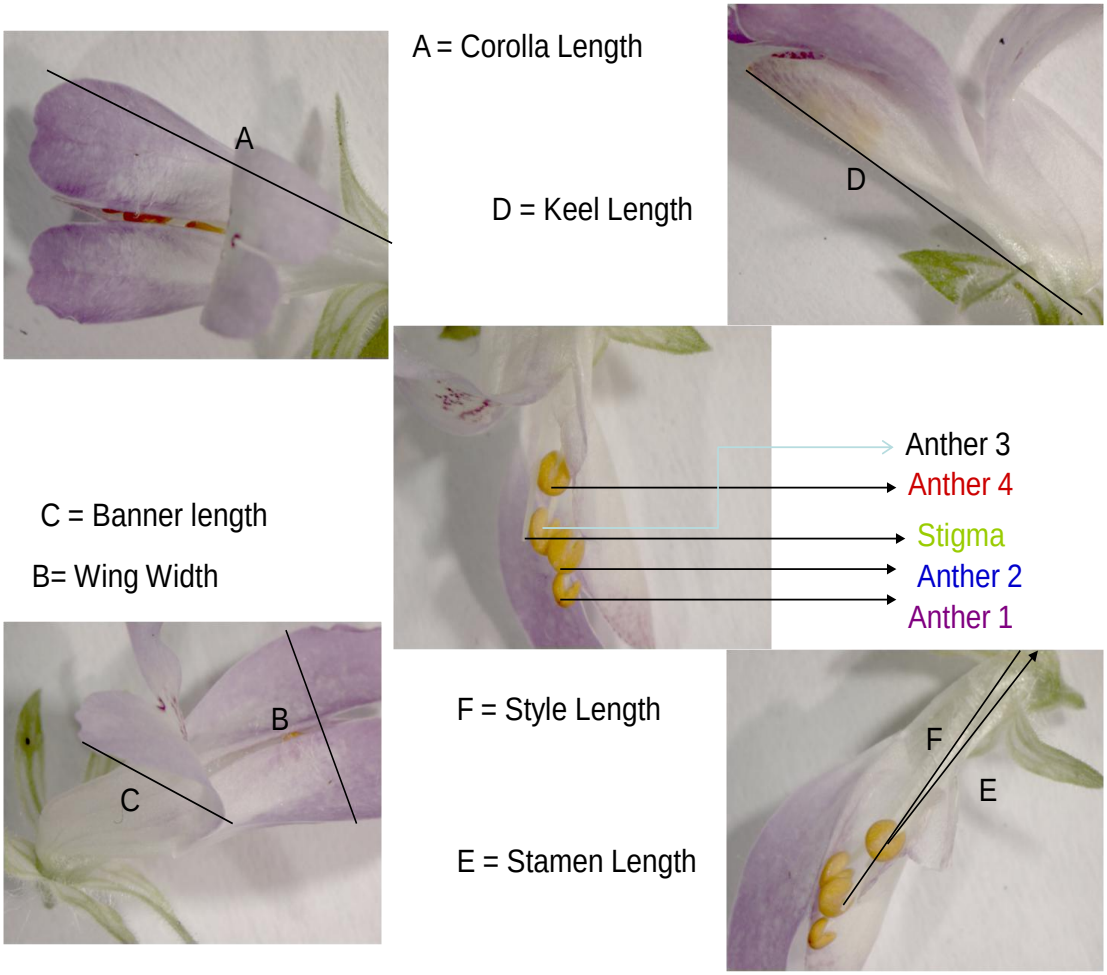


Figure 3.1 *Collinsia heterophylla* flower under the scanning electron showing the eight flower traits measured.

3.2.3.2 Genetic Variability and Heritability of Floral Traits

To examine if there are genetic variations and correlations as well as heritabilities in the floral traits studied, the genetic component of variation in these traits was assessed using the seeds obtained from Norway and Chiltern populations. Thirty pre-parental plants were randomly chosen from each population and were allowed to set seeds without any manipulations. It was assumed that the seeds set were selfed seeds,

although there were white and green flies present in the greenhouse during the flowering period, these insects are not known to pollinate *C. heterophylla*. In 2007, *Collinsia heterophylla* seeds from the two different populations (Norway and Chiltern) were grown in the greenhouse and were randomly crossed within each population to raise seeds for the parental generations. In 2008, plants with distinctly small floral size were selected from the Norway population and plants with large floral size were selected from the Chiltern population. These plants were used as the parental plants and the eight floral morphological traits were measured on these plants. Seeds obtained from these parental plants were sown and grown in the greenhouse of the University of Portsmouth in spring/summer of 2009; these were the F1 generations. At flowering, all the eight floral morphological traits were measured on these F1 generations (Norway and Chiltern populations). Data obtained from these measurements were used to estimate genetic variability.

To estimate narrow-sense heritability of the floral traits measured, crosses were carried out in 2008 on twenty parental plants each from the two populations. The twenty plants were randomly selected from each population. One plant from each population was selected to act as a donor as well as a recipient (i.e. sire and dam). Crosses were made between these donor plants and twenty other parent plants including the donor (acting as dams) within their populations. The donor plants acted as a donor and a recipient at the same time. Fully receptive flowers at stage three of floral development (i.e. 3rd to 4th day after anthesis) were carefully emasculated using a sharp pair of forceps and crosses were performed on the emasculated flowers by removing single dehisced anthers from the donor/sire with forceps and applying it directly to the stigma of the recipients/dams until the stigma is fully covered with pollen grains. Five to ten flowers on each individual plant were crossed using the single donor plant within each population. The rest of the flowers on each plant were left to open/self pollinate and used as control.

The seed capsules of the treatment (cross pollinated flower) were bagged a few days before they were ripe, in order to avoid losing any seeds; because *Collinsia heterophylla* capsule break open to disperse their seeds (explosive mechanism). They were not bagged earlier to ensure that the capsules were matured and did not suffer stress from bagging). The mature seeds were collected and stored away in the cold room for sowing in 2009. Five seeds from each set of parents were grown to flowering in separate flower pots in the greenhouse in February 2009. Transplanted seedlings that did not continue to grow or those that died within three weeks after planting were replanted. Thus the experimental design was 1 sire x 20 dams x 5 offspring/dam = 100 offspring x 2 populations = 200 offspring F1 generation. The actual number of offspring was 181, because not all offspring survived through to the flowering period despite all effort made. The eight floral traits of interest (see above) were measured in at least 3 offspring from each parental plant, hence total offspring measured = 60 plants/population).

3.2.3.3 Heritability Estimates

Heritability tells us only about variation within a particular population and it is the ratio of additive genetic variation to total phenotypic variation; it is not an absolute measure of genetic variation. Therefore it is important to note that environmental differences can cause phenotypic differences between populations, even if $h^2 = 0$.

The conventional method used to estimate heritability for a quantitative trait of interest is to take measurements directly on parents and offspring (Falconer, 1989; Lynch and Walsh, 1998; Albouyeh and Ritland, 2009); this is then followed by regression of offspring measurements on parent measurements. The slope of the regression is a measure of the heritability of the trait. For several species, parents and their offspring are easily identified in the field; however in plants, progeny can be sampled as seed. On the other hand, in many species including most plant species, only one parent can be recognized: the mother, while the male parent is an unknown pollen donor (Lynch and Walsh, 1998).

3.2.3.4 Parent-Offspring Regression

The parent–progeny regression, specifically the midparent–offspring design is one of four major designs for deducing the heritability of a quantitative trait; the other three are single parent–offspring, half-sib family, and full-sib family designs (Falconer, 1989; Albouyeh and Ritland, 2009). Parent–offspring regression is the most straightforward method for estimating heritability for three reasons: (i) it is possible to base the essential computations on least-squares regression, the statistical properties are well known; (ii) neither dominance nor linkage influences the covariance between parents and offspring and (iii) it is not biased when parents are selected on the basis of their phenotype (see Lynch and Walsh, 1998; reviewed in Albouyeh and Ritland, 2009).

Parent - Offspring designs compare phenotypic variance between parents and offspring and is one of the most commonly used methods. A specific phenotypic trait is measured for both the parent and the offspring at the same age and compared using regression, the slope of the regression of offspring on parents' estimates heritability - h^2. The mid-parent-offspring regression (i.e. the average of the two parents on offspring) gives an estimate of the total narrow-sense heritability (see Oosterhout and Brakefield, 1999; Ward, 2000; Keller *et al.*, 2001).

$$\mathbf{b}_{\mathbf{op}} = \frac{V_a}{V_p} = h^2$$

Formula for mid-parent offspring regression

Source: (Falconer, 1989; Lynch and Walsh, 1998)

A single parent-offspring is a comparison between either the mother or father and the offspring and gives an estimate of half of the narrow sense heritability. The slope of the father-offspring regression may differ from the slope of the mother-offspring regression due to maternal effects, a component of the environmental variance and could therefore be used to determine the effect of the maternal environment on a specific trait.

$$\mathbf{b}_{\mathbf{op}} = \frac{V_a}{2V_p} = \frac{1}{2}h^2$$

One parent - offspring formula

Source: (Falconer, 1989; Lynch and Walsh, 1998)

3.2.3.5 Heritability and Correlations of Anther–Stigma Contact (ASC)

To estimate the correlation and heritability of anther-stigma contact in *Collinsia heterophylla*, stamen and pistil lengths were measured in parental and offspring generations of the two populations studied in 2008 and 2009. The anther-stigma contact (the stage during anthesis when the stigma and the dehiscing anther came in contact -ASC) was measured in two ways in all parental and offspring generations used for the experiment. The first method was a direct visual observation of the floral developmental stage when stigma and anther came in contact with each other. If the stigma was in contact with the dehiscing anther, it was scored one (1) but if the stigma was not in contact with the dehiscing anther it was scored zero (0). The second method was an indirect method estimated from the measurement of stamen and pistil lengths using digital calipers, both lengths were measured in each of the five flowers per floral stage /individual plant). In 2007, the floral stage at which anthers and stigmas came into contact was estimated in the two populations studied, by observing at least two newly opened flowers per individual plant were monitored twice a day (every 12 h) until stage 4 was achieved. To be able to compare the two methods used to estimate timing of anther–stigma contact, pistil length in five flowers was also measured each day after flower opening in a subset of these plants (in total 5 x 5 = 25 flowers per plant). Logistic regression was used to calculate the anther-dehiscence stage at which 50% of the flowers had anthers and stigmas in contact (ASC-50) following Armbruster *et al.* (2002); see also Kalisz *et al.*, 1999). The sample unit was thus one value for each individual plant.

3.2.3.6 Stigma Receptivity

The timing of stigma receptivity was established in relation to pistil elongation at each of the five floral development stages in the two populations of *Collinsia heterophylla* grown in the greenhouse during the spring/summer of 2007. There are two different methods for testing stigma receptivity as described by Kalisz *et al.* (1999). The simplest is the test to determine the presence of peroxidases on the sigma of receptive stigmas, it is known as the stigmatic peroxides (SPA) method described by Kearns and Inouye (1993) (see Armbruster *et al.*, 2002). Kearns and Inouye (1993) described the reaction of receptive stigmas in 3% hydrogen peroxide solution as the vigorous release of bubbles when receptive and the absence of bubbles when not receptive. The second method is to observe and score the presence or absence of pollen tubes in the style of the flower (Kalisz *et al.*, 1999 and Stpiczynska, 2003).

The method adopted in this research was the peroxidases test. However, the percentage of hydrogen peroxide used in this research was 7% because there was no visible reaction when 3% was used. Stigmas were collected from flowers at five different floral developmental stages, starting from when the flower opened and through the four stages of anther dehiscence. Prior to the peroxidase test, flowers at each of the five developmental stages were identified in each plant and the styles from each flower were collected with a pair of forceps. These styles were placed in 7% hydrogen peroxide on glass slides and covered with cover slips; the styles in hydrogen peroxide solution were left for 2 – 3 minutes and observed for bubbles. Stigmas that produced bubbles within 2 - 3 minutes were considered receptive and the scoring for each stigma was either positive/1 (bubbles present) or negative/0 (bubbles absent). At least three flowers per developmental stage were observed for stigma receptivity in each plant and across the two populations studied (3 X 5 = 15 flowers/plant). The stigmas were used to calculate one (mean) value for the onset of stigma receptivity for each individual plant. The day at which 50% of the plants produced stigmas that tested positive for peroxidise activity (SPA-50) was calculated using logistic regression (see method used

by Armbruster *et al.*, 2002). The result obtained was similar to that observed by Armbruster *et al.* (2002).

3.3 Data Analysis

All phenotypic analyses (means, standard deviations, correlations) were carried out for all eight floral morphological traits measured in the twenty parental plants from each of the two populations. The analyses were performed on the average of five flower measurements, because floral morphological measurements were made on five flowers and replicated three times on each flower per individual plants in the two parental generations. All measurements were in millimetres to the nearest 0.01mm.

3.3.1 Floral-Traits Variations

In order to find out if there is genetic variation in floral traits of *Collinsia heterophylla,* analyses of additive genetic variances and correlations are carried out using the parental generation as well as the offspring generation. Prior to applying any statistical analysis tool to compare these variances, Kolmogorov-smirnov and Shapiro-Wilk tests for normality were carried out. The p-value obtained was higher than 0.05; therefore, the data was normally distributed. Similarly, a Levene's test for homogeneity of variances was computed using SPSS 16.0 (2008). The result obtained showed the p-value to be generally higher than 0.05; therefore we had no reason to reject homogeneity of variances, meaning that the homogeneity of variances has been satisfied (Appendix 1.0). This means that the data collected conformed to the criteria for parametric test and the analysis of variance; consequently, the variations in mean floral trait lengths measured were further assessed for significance using Analysis of Variance (ANOVA) in SPSS 16.0 (SPSS, 2008). Initially, a multivariate analysis of variance (General Linear Model – GLM) was performed to test for significant additive genetic variance over all traits simultaneously; subsequently, One-way analysis of variance was computed for each trait individually to observe how each trait varies in each plant and at each floral developmental stage. This variation was also

examined within and between populations studied. The result of the One-way analysis of variance was similar to the multivariate result, hence a parametric *post-hoc* Tukey test was conducted to reveal where the differences lay. Some of the traits were found to show correlations; therefore both the multivariate and One-way ANOVA were shown in the result.

3.3.2 Floral-Traits Correlations

To assess the correlations in the floral traits measured, Two-tailed Bivariate Pearson correlations were carried out on the parental generations of the two populations studied. Thereafter, in order to measure to what extent the floral traits are genetically correlated, Two-tailed Bivariate Pearson correlations were carried out using the floral traits values for the parents and offspring generations of the two populations. Furthermore, parent-offspring linear correlations were performed on all floral traits to estimate the phenotypic and additive genetic correlations among floral traits measured.

3.3.3 Floral-Traits Heritabilities

To measure the heritability of these floral traits and examine which of the trait will respond more to selection, narrow-sense heritabilities (h^2) were calculated using regression analyses: parent-offspring regression (midparent-offspring) was used to calculate the genetic component of the traits (Falconer and McKay, 1996; Lynch and Walsh, 1998). In each analysis, measurements of offspring of the same parent(s) were averaged. Midparent–offspring regressions usually estimate heritabilities directly, whereas single parent–offspring regression estimates half of the heritability (Lynch and Walsh, 1998). Although the father was constant, the midparent-regression was used following Lynch and Walsh (1998), as this gives the best estimate for narrow-sense heritability. To minimize sampling error due to the small number of offspring measured, weighted least-square (WLS) regressions was used for heritability estimates, following Lynch and Walsh (1998); pp. 539–542. However, the results of

the WLS regressions were very similar to those obtained from linear regression; therefore, the midparent linear regression was presented in the results and discussions.

3.4 Results

3.4.1 Patterns of Floral-Traits Variations

The mean, standard deviations, standard error of mean, variance, minimum and maximum values for all the traits measured is shown in Table 3.1. In the small-flowered Norway population, floral traits measured in millimetres vary as follows: (corolla length 13.96-19.40, banner length 5.28-7.61, wing width 6.12-10.28, keel length 13.40-18.89, stamen1 length 10.51-16.79, pistil 8.12-15.85, stamen3 length 7.78-17.03); while in the large-flowered Chiltern population, floral traits variations measured in millimetres are: (corolla length = 16.62-25.35, banner length = 6.09-10.98, wing width = 7.57-15.85, keel length = 15.33-23.35, stamen1length = 11.68-21.37, pistil = 8.87-19.58, stamen3 length = 9.11-18.20).

Table 3.1 Descriptive statistics from a pooled data of seven out of eight floral traits measured (i.e. corolla length, wing width, banner length, keel length, 1st stamen and 3rd stamen lengths, and pistil length) in *C. heterophylla* (Norway and Chiltern) populations; showing the mean, standard deviations (Std. D), standard error of means ($S.E_M$) variance components (V), minimum (Min), maximum (Max) and range.

Population		Corolla L	WingW	Banner L	KeelL	Stamen 1L	PistilL	Stamen 3L
Norway	Mean	17.541	8.694	6.463	16.339	14.439	12.372	12.499
	Std. D	1.360	1.007	0.541	1.369	1.718	2.240	2.675
	S. E_M	0.136	0.101	0.054	0.137	0.172	0.224	0.267
	V	1.850	1.014	0.293	1.873	2.952	5.018	7.155
	Min.	13.956	6.123	5.276	13.396	10.513	8.123	7.781
	Max.	19.396	10.279	7.609	18.889	16.793	15.852	17.033
Chiltern	Mean	20.890	12.241	8.0950	19.154	16.180	13.802	13.498
	Std. D	2.119	1.734	1.036	1.958	2.087	2.968	2.520
	S. EM	0.212	0.173	0.104	0.196	0.209	0.297	0.252
	V	4.489	3.006	1.073	3.834	4.356	8.805	6.346
	Min.	16.621	7.567	6.092	15.334	11.675	8.868	9.111
	Max.	25.347	15.852	10.984	23.350	21.368	19.577	18.203

3.4.2 Between-Populations Floral-Traits Variations

The Multivariate ANOVA of a pooled data from the parental generation of Chiltern and Norway populations shows highly significant overall effects of plants, stage and populations on all traits measured (Table 3.2). Results obtained from the General Linear Model (GLM) Wilks' Lambda Multivariate tests in Table 3.2 revealed that for the eight floral traits measured, the effect of plants was significant (F_8 = 2.438, P = 0.016); the effect of floral developmental stages and population were also significant (stage F_{32} = 25.870, P < 0.001; population F_1 = 19.569, P < 0.001).

Table 3.2 General linear Model Wilks' Lambda Multivariate tests from a pooled dada for the two populations of *Collinsia heterophylla* (Norway and Chiltern) using the eight floral traits as dependent variables with Population and stages as fixed factors, and plant as covariates. Plant and population df = 8, stage and Pop*stage df = 32.

Effect	*Wilks' Lambda Value*	*F*	*P*
Plant	0.903	2.438[a]	= 0.016
Population	0.538	19.569[a]	<0.001
Stage	0.052	25.870	<0.001
*Population * Stage*	0.496	4.398	<0.001

The results of the One-way ANOVA (Tables 3.3, 3.4, and 3.5) performed on the pooled data from the parental generations in the two populations studied showed that floral traits variations among plants was significantly different in five of the eight floral traits measured namely: corolla length, wing width, banner length, keel length and stamen1 length; with df = 39, 199 and P = 0.001. But there was no significant variation among plants in pistil length ($F_{39, 199}$ = 0.793, P = 0.801); stamen 3 length ($F_{39, 199}$ = 0.558, P = 0.983) and anther-stigma contact ($F_{39, 199}$ = 0.621, P = 0.959) (Table 3.3). The variations in floral traits across the five floral developmental stages showed significant different effect in seven of the

eight floral traits measured with df = 4, 199 and P < 0.001, except for the banner length where $F_{4, 199} = 4.113$, P = 0.003 (Table 3.4).

Table 3.3 One-way ANOVA to explain the variation in floral traits among all plants measured in the two populations of *Collinsia heterophylla* (Norway and Chiltern parental generation), using the eight floral traits as dependent variables and plant as the factor; df = 39, 199.

Sources of Variation		*Sum of Squares*	*Mean Square*	*F*	*P*
Corolla length * plant	Between Groups	773.456	19.832	7.652	<0.001
	Within Groups	414.660	2.592		
	Total	1188.116			
Wing width * plant	Between Groups	855.121	21.926	20.404	<0.001
	Within Groups	171.932	1.075		
	Total	1027.053			
Banner length * plant	Between Groups	237.712	6.095	31.751	<0.001
	Within Groups	30.715	0.192		
	Total	268.427			
Keel length * plant	Between Groups	617.989	15.846	7.386	<0.001
	Within Groups	343.285	2.146		
	Total	961.274			
Stamen1 length * plant	Between Groups	286.700	7.351	1.999	=0.001
	Within Groups	588.364	3.677		
	Total	875.064			
Pistil length * plant	Between Groups	238.157	6.107	0.793	=0.801
	Within Groups	1232.647	7.704		
	Total	1470.804			
Stamen3 length * plant	Between Groups	166.072	4.258	0.558	=0.983
	Within Groups	1220.499	7.628		
	Total	1386.571			
ASC * plant	Between Groups	5.590	0.143	0.621	=0.959
	Within Groups	36.944	0.231		
	Total	42.534			

Table 3.4 One-way ANOVA to explain the variation in floral traits across all the five floral developmental stages measured in the two populations of *Collinsia heterophylla* (Norway and Chiltern parental generations), using the eight floral traits as dependent variables and stage as factor; df = 4 and 199.

Sources of Variation		*Sum of Squares*	*Mean Square*	*F*	*P*
Corolla length * stage	Between Groups	347.132	86.783	20.123	<0.001
	Within Groups	840.984	4.313		
	Total	1188.116			
Wing width * stage	Between Groups	139.111	34.778	7.638	<0.001
	Within Groups	887.942	4.554		
	Total	1027.053			
Banner length * stage	Between Groups	20.883	5.221	4.113	=0.003
	Within Groups	247.544	1.269		
	Total	268.427			
Keel length * stage	Between Groups	296.376	74.094	21.730	<0.000
	Within Groups	664.898	3.410		
	Total	961.274			
Stamen1 length * stage	Between Groups	515.489	128.872	69.888	<0.001
	Within Groups	359.576	1.844		
	Total	875.064			
Pistil length * stage	Between Groups	1108.931	277.233	149.390	<0.001
	Within Groups	361.873	1.856		
	Total	1470.804			
Stamen3 length * stage	Between Groups	1095.148	273.787	183.199	<0.001
	Within Groups	291.424	1.494		
	Total	1386.571			
ASC * stage	Between Groups	27.759	6.940	91.591	<0.001
	Within Groups	14.775	0.076		
	Total	42.534			

Similarly, the effects of populations on floral-traits variations were significant for seven of the eight floral traits measured (df = 1, 199; P < 0.001), while there is no significant difference in anther-stigma variation between the two populations ($F_{1, 199} = 2.078$, P = 0.151) (Table 3.5).

Table 3.5 One-way ANOVA showing variations in the eight floral traits measured between and within the two populations of *Collinsia heterophylla* (Norway and Chiltern parental generations), using the eight floral traits as dependent variables and population as the factor; df = 1 and 199.

Sources of Variation		*Sum of Squares*	*Mean Square*	*F*	*P*
Corolla length * Population	Between Groups	560.638	560.638	176.909	<0.001
	Within Groups	627.478	3.169		
	Total	1188.116			
Wing width * Population	Between Groups	629.090	629.090	312.993	<0.001
	Within Groups	397.963	2.010		
	Total	1027.053			
Banner length * Population	Between Groups	133.213	133.213	195.070	<0.001
	Within Groups	135.214	0.683		
	Total	268.427			
Keel length * Population	Between Groups	396.275	396.275	138.872	<0.001
	Within Groups	564.999	2.854		
	Total	961.274			
Stamen1 length * Population	Between Groups	151.545	151.545	41.472	<0.001
	Within Groups	723.519	3.654		
	Total	875.064			
Pistil length * Population	Between Groups	102.325	102.325	14.805	<0.001
	Within Groups	1368.479	6.912		
	Total	1470.804			
Stamen3 length * Population	Between Groups	49.989	49.989	7.405	=0.007
	Within Groups	1336.583	6.750		
	Total	1386.571			
ASC * Population	Between Groups	0.442	0.442	2.078	=0.151
	Within Groups	42.092	0.213		
	Total	42.534			

The results obtained from analysis of variance for pooled data of the parental generations of the two populations of *C. heterophylla* studied showed a very similar pattern to that obtained from the pooled data of the

offspring generations. Furthermore, as a result of the significant floral traits variations observed between the two populations, one-way ANOVA was computed to examine how floral traits varied within-population.

3.4.3 Within-Population Floral-Traits Variations

The One-way ANOVA for floral-traits variations among the parental plants of the Norway population showed that there was no significant variations in seven of the eight floral traits measured, but the banner length showed significant variation among plants; $F_{19, 99} = 3.904$, $P < 0.001$ (Table 3.6). However, the results obtained for floral-traits variations across floral developmental stages in the Norway population showed significant variations in all floral-traits measured across the five stages; df = 4 and 99, $P < 0.001$ (Table 3.7).

The result obtained from the *post hoc* Tukey HSD test among floral developmental stages (0 to 4) in the Norway population parental plants showed that corolla length at stage 0 was significantly different from corolla length at stages 1, 2, 3, and 4 ($P < 0.001$). Corolla length at stage 1 also significantly differs from corolla length at stages 3 and 4 ($P < 0.001$), but difference between stages 1 and 2 was marginal ($p = 0.045$). There was no significant difference in corolla length between stages 2 and 3 ($P = 0.613$), while stages 3 and 4 showed marginal difference ($P = 0.023$). The wing width at stage 0 showed significant difference from at stages 1, 2, 3 and 4, $P < 0.001$; while stage 2 did not differ significantly from stages 1, 3 and 4. Similarly, the banner length at stage 0 showed significant difference from banner lengths at stages 1, 2, 3 and 4 ($P < 0.001$); but no significant difference between banner length at stage 2 and at stages 1, 3 and 4. In addition, the keel length at stage 0 is significantly different from at stages 1, 2, 3 and 4 ($P < 0.001$). Also keel length at stage 1 differed significantly from at stage 3 and 4, and keel length at stage 2 is different from at stage 4; but no significant difference between stages 3 and 4.

Furthermore, the *post hoc* Tukey test showed that there is significant difference in stamen lengths across the five floral developmental stages; first and third stamen lengths at stage 0 differ from

at stages 1, 2, 3 and 4 (P < 0.001). However, the third stamen length at stage 1 did not differ significantly from at stage 2 (P = 0.096). Finally, pistil lengths showed significant difference across all the five floral developmental stages (p < 0.001). Also, the anther-stigma contact (ASC) at stage 0 differed significantly from at stages 2, 3 and 4(P = 0.001), but no significant difference between stages 0 and 1, stages 1 and 2, as well as stages 2 and 3 (Appendix 1.6).

Table 3.6 One-way ANOVA showing variations in the eight floral traits measured among plants within the Norway parental population of *Collinsia heterophylla*, using the eight floral traits as dependent variables and plants as factors; the df = 19 and 99.

Sources of variation		*Sum of Squares*	*Mean Square*	*F*	*P*
Corolla length * Plant	Between Groups	16.632	0.875	0.421	=0.982
	Within Groups	166.472	2.081		
	Total	183.104			
Wing width * Plant	Between Groups	28.077	1.478	1.634	=0.068
	Within Groups	72.333	0.904		
	Total	100.410			
Banner length * Plant	Between Groups	13.936	0.733	3.904	<0.001
	Within Groups	15.029	0.188		
	Total	28.964			
Keel length * Plant	Between Groups	23.402	1.232	0.608	=0.890
	Within Groups	162.037	2.025		
	Total	185.439			
Stamen1 length * Plant	Between Groups	25.572	1.346	0.404	=0.986
	Within Groups	266.663	3.333		
	Total	292.235			
Pistil length * Plant	Between Groups	12.111	0.637	0.105	=1.000
	Within Groups	484.634	6.058		
	Total	496.745			
Stamen3 length * Plant	Between Groups	59.831	3.149	0.388	=0.989
	Within Groups	648.531	8.107		
	Total	708.362			
ASC * Plant	Between Groups	4.008	0.211	1.091	=0.376
	Within Groups	15.472	0.193		
	Total	19.480			

Table 3.7 One-way ANOVA showing variations in the eight floral traits measured across floral developmental stages within the Norway parental population of *Collinsia heterophylla*, using the eight floral traits as dependent variables and stages as factors; the df = 4 and 99.

Sources of variation		*Sum of Squares*	*Mean Square*	*F*	*P*
Corolla length * Stage	Between Groups	143.423	35.856	85.842	<0.001
	Within Groups	39.681	0.418		
	Total	183.104			
Wing width * Stage	Between Groups	58.650	14.663	33.356	<0.001
	Within Groups	41.759	0.440		
	Total	100.410			
Banner length * Stage	Between Groups	12.060	3.015	16.943	<0.001
	Within Groups	16.905	0.178		
	Total	28.964			
Keel length * Stage	Between Groups	141.985	35.496	77.603	<0.001
	Within Groups	43.454	0.457		
	Total	185.439			
Stamen1 length * Stage	Between Groups	241.466	60.366	112.959	<0.001
	Within Groups	50.769	0.534		
	Total	292.235			
Pistil length * Stage	Between Groups	459.965	114.991	297.013	<0.001
	Within Groups	36.780	0.387		
	Total	496.745			
Stamen3 length * Stage	Between Groups	583.776	145.944	111.286	<0.001
	Within Groups	124.586	1.311		
	Total	708.362			
ASC * Stage	Between Groups	11.692	2.923	35.655	<0.001
	Within Groups	7.788	0.082		
	Total	19.480			

In the Chiltern population, the One-way ANOVA for floral traits variations among the parental plants showed that there is significant variations in the corolla length, wing width, banner length and keel length (P = 0.001), but there was no significant variations in stamen lengths, pistil length and anther-stigma contact (Table 3.8). The results obtained

for floral traits variations across floral developmental stages showed that seven floral traits vary significantly across the five stages (df = 4 and 99, P < 0.001; Table 3.9), except for banner length (F = 2.203, P = 0.074).

Table 3.8 One-way ANOVA showing variations in the eight floral traits measured among plants within the Chiltern parental population of *Collinsia heterophylla*, using the eight floral traits as dependent variables and plants as factors; the df = 19 and 99.

Sources of variation		***Sum of Squares***	***Mean Square***	***F***	***P***
Corolla length * Plant	Between Groups	196.186	10.326	3.328	<0.001
	Within Groups	248.188	3.102		
	Total	444.374			
Wing width * Plant	Between Groups	197.954	10.419	8.368	<0.001
	Within Groups	99.600	1.245		
	Total	297.554			
Banner length * Plant	Between Groups	90.563	4.766	24.308	<0.001
	Within Groups	15.687	0.196		
	Total	106.250			
Keel length * Plant	Between Groups	198.312	10.437	4.607	<0.001
	Within Groups	181.248	2.266		
	Total	379.561			
Stamen1 length * Plant	Between Groups	109.583	5.768	1.434	=0.135
	Within Groups	321.701	4.021		
	Total	431.284			
Pistil length * Plant	Between Groups	123.722	6.512	0.696	=0.812
	Within Groups	748.013	9.350		
	Total	871.735			
Stamen3 length * plant	Between Groups	56.253	2.961	0.414	=0.984
	Within Groups	571.968	7.150		
	Total	628.221			
ASC * Plant	Between Groups	1.140	0.060	0.224	=1.000
	Within Groups	21.472	0.268		
	Total	22.612			

Table 3.9 One-way ANOVA showing variations in the eight floral traits measured across the floral developmental stages within the Chiltern parental population of *Collinsia heterophylla*, using the eight floral traits as dependent variables and stages as factors; the df = 4 and 99.

Sources of variation		***Sum of Squares***	***Mean Square***	***F***	***P***
Corolla length * Stage	Between Groups	208.514	52.128	20.996	<0.001
	Within Groups	235.860	2.483		
	Total	444.374			
Wing width * Stage	Between Groups	81.938	20.484	9.025	<0.001
	Within Groups	215.616	2.270		
	Total	297.554			
Banner length * Stage	Between Groups	9.019	2.255	2.203	=0.074
	Within Groups	97.231	1.023		
	Total	106.250			
Keel length * Stage	Between Groups	155.850	38.962	16.546	<0.001
	Within Groups	223.711	2.355		
	Total	379.561			
Stamen1 length * Stage	Between Groups	280.994	70.248	44.405	<0.001
	Within Groups	150.290	1.582		
	Total	431.284			
Pistil length * Stage	Between Groups	679.207	169.802	83.786	<0.001
	Within Groups	192.528	2.027		
	Total	871.735			
Stamen3 length * Stage	Between Groups	532.813	133.203	132.633	<0.001
	Within Groups	95.408	1.004		
	Total	628.221			
ASC * Stage	Between Groups	18.486	4.622	106.411	<0.001
	Within Groups	4.126	0.043		
	Total	22.612			

The *post hoc* Tukey HSD test computed for floral developmental stages (0 to 4) in the Chiltern population parental generation showed that corolla length at stage 0 differed significantly from corolla length at stages

1, 2, 3, and 4 (P < 0.001). Also corolla length at stage 1 significantly differed from corolla length at stages 3 and 4 (P < 0.001). The wing width showed significant difference between stage 0 and stages 2, 3 and 4 (P < 0.001); while there was no significant difference in banner lengths across all developmental stages. In addition, the keel length at stage 0 was significantly different from at stages 1, 2, 3 and 4 (P < 0.001), and also keel length at stage 1 differed significantly from at stage 3 and 4. The *post hoc* Tukey test also showed that there is significant difference in stamen1 length between stage 0 and stages 1, 2, 3 and 4. Besides, stamen 1 length at stage 1 differed significantly from at stages 3 and 4, and stage 2 stamen 1 length was different from stage 4 (P < 0.001). The pistil length differed significantly across the five floral developmental stages; similarly the stamen 3 length differs significantly across floral developmental stages, but for stages 3 and 4 that showed no significant difference (P = 0.089). The anther-stigma contact (ASC) also differed significantly across all floral developmental stages, except for stages 3 and 4 that showed no significant difference (p = 0.974) [Appendix 1.7].

3.4.2 Mean Offspring and Midparent Descriptive Statistics

The mean, standard deviation, standard error of mean and variance for mean offspring and midparent values of all the traits measured are shown in Tables 3.10 and 3.11. Generally, the mean floral traits values in the Chiltern population were higher than in the Norway population.

Table 3.10 Mean offspring and midparent-offspring means, standard deviations (SD), standard error of means ($S.E_N$), variance (V) for the eight floral morphological traits measured in Norway population of *Collinsia heterophylla*. Total for the parental generation (*N = 20*) and total for the offspring generation (*N = 60*); floral traits measured in mm, anther-stigma contact (ASC) is a ordinal variable

Floral traits measured (mm)	Mean		Std. Deviation (SD)	Variance (V)
	Statistic	Std. Error		
Mean offspring corolla length	18.072	0.134	1.3416	1.800
Midparent corolla length	17.541	0.136	1.360	1.850
Mean offspring wing width	9.245	0.114	1.144	1.308
Midparent wing width	8.694	0.101	1.007	1.014
Mean offspring banner length	6.551	0.039	0.390	0.152
Midparent banner length	6.463	0.054	0.541	0.293
Mean offspring keel length	16.699	0.123	1.233	1.521
Midparent keel length	16.339	0.137	1.369	1.873
Mean offspring stamen1 length	14.622	0.155	1.547	2.393
Midparent stamen1 length	14.439	0.172	1.718	2.952
Mean offspring pistil length	12.510	0.226	2.255	5.085
Parent pistil length	12.372	0.224	2.240	5.018
Mean offspring stamen3 length	12.587	0.232	2.323	5.396
Parent stamen3 length	12.499	0.268	2.675	7.155
Mean offspring ASC	0.394	0.039	0.392	0.153
Parent ASC	0.380	0.044	0.444	0.197

Table 3.11 Mean offspring and midparent-offspring means, standard deviations (SD), standard error of means ($S.E_N$), variance (V) for the eight floral morphological traits measured in Chiltern population of *Collinsia heterophylla*. Total for the parental generation (*N = 20*) and total for the offspring generation (*N = 60*); floral traits measured in mm, anther-stigma contact (ASC) is a ordinal variable

Floral Traits measured (mm)	Mean		Std. Deviation (SD)	Variance (V)
	Statistic	Std. Error		
Mean offspring corolla length	21.192	0.179	1.786	3.190
Parent corolla length	20.890	0.212	2.119	4.489
Mean offspring wing width	12.652	0.134	1.339	1.794
Parent wing width	12.241	0.173	1.734	3.006
Mean offspring banner length	8.550	0.068	0.683	0.467
Parent banner length	8.095	0.104	1.036	1.073
Mean offspring keel length	19.486	0.163	1.626	2.643
Parent keel length	19.154	0.196	1.958	3.834
Mean offspring stamen1 length	16.268	0.171	1.712	2.932
Parent stamen1 length	16.180	0.209	2.087	4.356
Mean offspring pistil length	14.331	0.300	2.998	8.986
Parent pistil length	13.802	0.297	2.967	8.805
Mean offspring stamen3 length	13.475	0.255	2.548	6.490
Parent stamen length	13.498	0.252	2.519	6.346
Mean offspring ASC	0.463	0.044	0.442	0.195
Parent ASC	0.474	0.048	0.478	0.228

3.4.3 Floral Phenotypic and Genetic Correlations

The phenotypic correlations among floral traits in the parental generations for the two populations of *C. heterophylla* are shown on Table 3.12 and Table 3.13. Results on Table 3.14 and Table 3.15 show the phenotypic correlations among all eight floral traits in the offspring

generations of *C. heterophylla*. All the eight floral morphological traits measured in both the parental and the offspring generations for the two populations studied showed positive phenotypic correlation; and most of the traits showed moderate to high correlations in the two parental generations (Norway = 0.426 to 0.953, Chiltern = 0.237 to 0.980, as well as in the offspring generations (Norway = 0.540 to 0.968; Chiltern = 0.576 to 0.986).

Table 3.12 Phenotypic correlations in *Collinsia heterophylla* (Norway parental generation), calculated as the Pearson correlations (2-tailed) among the eight floral traits measured in the parental generation (N = 20). Using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**). Traits measured are corolla length (Corll), wing width (Wingw), banner length (Bannerl), keel length (Keell), stamen1 length (Stamen1l), pistil length (Pistil l), stamen3 length (Stamen3l), and anther-stigma contact (ASC).

Traits	**Corll**	**Wingw**	**Bannerl**	**Keell**	**Stamen1 l**	**Pistil l**	**Stamen3 l**	**ASC**
Corll	1							
Wingw	0.748**	1						
Bannerl	0.575**	0.737**	1					
Keell	0.923**	0.701**	0.406**	1				
Stamen1l	0.928**	0.743**	0.453**	0.953**	1			
Pistill	0.900**	0.766**	0.561**	0.934**	0.934**	1		
Stamen3l	0.823**	0.581**	0.662**	0.753**	0.758**	0.861**	1	
ASC	0.588**	0.426**	0.677**	0.448**	0.447**	0.609**	0.868**	1

** Correlation is significant at the 0.01 level (2- tailed)

However, the results obtained shows clearly that in the two populations studied, the corolla appears to have a very high correlation with the stamens, keel and pistil. The correlations between corolla and the keel are expected as the keel is also a petal, and hence part of the corolla (Tables 3.12 and 3.13). In the Norway populations, the highest correlation is seen between the keel and stamen1 (r = 0.928 – Table 3.12); while the highest correlation in the Chiltern population was recorded between the corolla and the keel (r = 0.980), followed by corolla and stamen1 L (r = 0.914 – Table 3.13)

Table 3.13 Phenotypic correlations in *Collinsia heterophylla* (Chiltern parental generation), calculated as the Pearson correlations (2-tailed) among the eight floral traits measured in the parental generation (N = 20). Using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**) and *P*-value < 0.05 are judged significant (*).Traits measured are corolla length(Corll), wing width(Wingw), banner length(Bannerl), keel length(Keell), stamen1 length(Stamen1l), pistil length(Pistil l), stamen3 length(Stamen3l), anther-stigma contact(ASC).

Traits	**Corll**	**Wingw**	**Bannerl**	**Keell**	**Stamen1 l**	**Pistil l**	**Stamen3 l**	**ASC**
Corll	1							
Wingw	0.796**	1						
Bannerl	0.569**	0.794**	1					
Keell	0.980**	0.782**	0.596**	1				
Stamen1l	0.914**	0.698*	0.496**	0.914**	1			
Pistill	0.766**	0.549**	0.379**	0.764**	0.855**	1		
Stamen3l	0.830**	0.585**	0.332**	0.800**	0.908**	0.896**	1	
ASC	0.537**	0.398**	0.237*	0.511**	0.632**	0.824**	0.801**	1

** Correlation is significant at the 0.01 level (2- tailed)

* Correlation is significant at the 0.05 level (2- tailed)

The results obtained from the floral morphological traits correlations for the offspring generations of Norway and Chiltern populations show a similar pattern to those of the parental generations. In the Norway population, the stamen1 shows the highest correlation with the corolla (r = 0.952) [Table 3.14]; while in the Chiltern population, the Stamen1 and stamen3 showed the highest correlation with the corolla (r = 958), followed by the keel and stamen1 (r = 935) [Table 3.15]. Generally, all floral morphological traits measured in the offspring generations showed higher values for correlations than the parental generations.

Table 3.14 Phenotypic correlations in *Collinsia heterophylla* (Norway population offspring generation), calculated as the Pearson correlations (2-tailed) among the eight floral traits measured in the offspring generation (N = 60). Using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**).Traits measured are corolla length, wing width, banner length, keel length, stamen1 length, pistil length, stamen3 length, anther-stigma contact(ASC).

Floral Traits	Corolla length	Wing width	Banner length	Keel length	Stamen1 length	Pistil length	Stamen3 length	ASC
Corolla length	1							
Wing width	.837**	1						
Banner length	.793**	.853**	1					
Keel length	.968**	.744**	.718**	1				
Stamen1 length	.911**	.673**	.746**	.952**	1			
Pistil length	.841**	.619**	.720**	.903**	.942**	1		
Stamen3 length	.827**	.601**	.757**	.875**	.908**	.951**	1	
ASC	.664**	.540**	.673**	.682**	.718**	.845**	.895**	1

****. Correlation is significant at the 0.01 level (2-tailed).**

The results of the genetic correlations in floral morphological traits between parent and offspring generations are shown in Table 3.16 (Norway population) and Table 3.17 (Chiltern population). Generally, the additive genetic correlations among the floral morphological traits measured in the Norway population were similar to the phenotypic correlations (Tables 3.12, 3.14, and 3.16). In the Norway population, the results obtained for the genotypic correlations in the floral morphological traits between parent and offspring generations shown in Table 3.16 are similar to that obtained in the Norway offspring population (Table 3.14).

Table 3.15 Phenotypic correlations in *Collinsia heterophylla* (Chiltern population offspring generation), calculated as the Pearson correlations (2-tailed) among the eight floral traits measured in the offspring generation (N = 60). Using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**).Traits measured are; corolla length, wing width, banner length, keel length, stamen1 length, pistil length, stamen3 length, anther-stigma contact(ASC).

Floral traits	Corolla length	Wing width	Banner length	Keel length	Stamen1 length	Pistil length	Stamen3 length	ASC
Corolla length	1							
Wing width	.907**	1						
Banner length	.697**	.838**	1					
Keel length	.986**	.908**	.702**	1				
Stamen1 length	.953**	.841**	.623**	.935**	1			
Pistil length	.870**	.768**	.653**	.868**	.890**	1		
Stamen3 length	.920**	.813**	.602**	.907**	.958**	.954**	1	
ASC	.735**	.673**	.576**	.729**	.772**	.919**	.889**	1

****. Correlation is significant at the 0.01 level (2-tailed).**

In contrast to the results obtained in the Chiltern population, the genetic correlation coefficients in Norway population were generally higher than in the Chiltern population. For example, all eight floral traits measured in the Norway population were significantly correlated at 0.01 CI; with correlation coefficients ranging from r = 0.416 to r = 0.937 (Table 3.16). Although the parent banner length showed lower correlations than other traits, the correlation coefficients obtained were higher in the Norway population than those obtained in the Chiltern population (compare Table 3.16 and 3.17). In addition, in the Norway population, the highest correlation coefficients were found in the corolla, stamens and pistil (ranging from r = 0.602 to r = 0.937) [Table 3.16].

Table 3.16 Pearson's 2-tailed correlations test, showing the correlations between parents and offspring generation for the eight floral traits in the Norway population of *Collinsia heterophylla*. The values represent correlation coefficients. The parental generation N = 20 and the offspring generation N = 60, and using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**).

Midparent Floral Traits	Mean Offspring Floral Traits							
	Corolla length	Wing width	Banner length	Keel length	Stamen1 length	Pistil length	Stamen3 length	ASC
Corolla length	0.776**	0.634**	0.708**	0.797**	0.854**	0.834**	0.812**	0.702**
Wing width	0.606**	0.470**	0.576**	0.642**	0.714**	0.709**	0.692**	0.599**
Banner length	0.507**	0.416**	0.518**	0.543**	0.598**	0.620**	0.588**	0.509**
Keel length	0.755**	0.564**	0.673**	0.783**	0.835**	0.838**	0.832**	0.738**
Stamen1 length	0.774**	0.587**	0.690**	0.805**	0.878**	0.841**	0.824**	0.695**
Pistil length	0.800**	0.602**	0.734**	0.850**	0.905**	0.937**	0.922**	0.820**
Stamen3 length	0.720**	0.551**	0.665**	0.779**	0.815**	0.879**	0.867**	0.792**
ASC	0.546**	0.435**	0.510**	0.592**	0.615**	0.696**	0.696**	0.667**

**. Correlation is significant at the 0.01 level (2-tailed).

However, it was observed that in the Chiltern population, using Pearson's correlations 2-tailed test, there was significant correlations among seven floral traits measured in the parental and offspring generations; the correlation coefficient values rang from r = 0.214 to r = 0.878 (Table 3.17). But parent banner length did not show significant correlations with the offspring generation (r = 0.042, P = 0.679). Also the correlations between the parent's banner length and all the eight floral morphological traits measured in the offspring showed the lowest correlations value (Table 3.17). For example, the correlations between parent banner length and offspring pistil length; and parent banner length and anther-stigma contact showed marginal significance with Pearson's correlations $r_{(banner\ x\ pistil)} = 0.214$ and $r_{(banner\ x\ ASC)} = 0.227$ (Table 3.17).

Table 3.17 Pearson's 2-tailed correlations test, showing the correlations between parents and offspring generation among the eight floral traits in the Chiltern population of *Collinsia heterophylla*. The values represent correlation coefficients (r). The parental generation N = 20 and the offspring generation N = 60, and using a table-wide significance level of 0.01 CI, individual *P*-value < 0.01 are judged significant (**).

Midparent Floral Traits	Mean Offspring Floral Traits							
	Corolla length	Wing width	Banner length	Keel length	Stamen1 length	Pistil length	Stamen3 length	ASC
Corolla length	0.531**	0.541**	0.369**	0.509**	0.561**	0.570**	0.596**	0.593**
Wing width	0.427**	0.459**	0.316**	0.398**	0.415**	0.408**	0.453**	0.444**
Banner length	0.303**	0.258**	0.042	0.299**	0.274**	0.214*	0.282**	0.227*
Keel length	0.504**	0.513**	0.321**	0.492**	0.526**	0.533**	0.563**	0.562**
Stamen1 length	0.663**	0.620**	0.409**	0.632**	0.704**	0.690**	0.733**	0.697**
Pistil length	0.753**	0.715**	0.513**	0.743**	0.769**	0.825**	0.839**	0.815**
Stamen3 length	0.748**	0.676**	0.454**	0.712**	0.810**	0.823**	0.870**	0.827**
ASC	0.736**	0.661**	0.491**	0.722**	0.746**	0.833**	0.850**	0.878**

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Generally, the results of genetic correlations shown in Tables 3.16 and 3.17 makes it clear that all floral traits in *Collinsia heterophylla* are correlated irrespective of the flower size and mating system exhibited by the population. However, the corolla, stamens and pistils were found to be more highly correlated than other floral traits; hence suggesting a likely linkage in the heredity of these four floral traits which are determinant of the mating system type.

3.4.4 Midparent-Offspring Regression Analyses

Regression analyses were carried out for the eight floral morphological traits to assess the linear relationships between the parents and the offspring in the two populations studied. The results obtained (Tables 3.18 and 3.19) show that there are very high significant linear

relationships for all eight floral traits in the Norway population (df = 1, 98; P < 0.001) [Table 3.18]. However in the Chiltern population, seven of the eight floral morphological traits showed very high linear relationships (df = 1, 98; P < 0.001), while there was no linear relationship between parent and offspring for banner length ($F_{1, 98}$ = 0.172, P = 0.679) [Table 3.19; Appendix 1.10].

Table 3.18 Simple Linear Regressions of offspring floral traits on midparent floral traits in *Collinsia heterophylla* (Norway population). Regression equation (*Y* = intercept + slope *x or y* = c + b*x*); slope of regression (bold print) = heritability R^2 = coefficient of determination and regression ANOVA with df = 1, 98.

Trait	Regression equation (Slope in bold)	R	R^2	F	P
Corolla length	y=4.649+**0.765**x	0.776	0.602	148.081	<0.001
Wing width	y=4.602+**0.534**x	0.470	0.221	27.822	<0.001
Banner length	y=4.139+**0.373**x	0.518	0.268	35.863	<0.001
Keel length	y=5.172+**0.705**x	0.783	0.613	155.249	<0.001
Stamen1 length	y=3.205+**0.791**x	0.878	0.771	330.245	<0.001
Pistil length	y=0.838+**0.944**x	0.937	0.878	707.997	<0.001
Stamen3 length	y=3.179+**0.753**x	0.867	0.751	296.181	<0.001
Anther-stigma contact	y=0.170+**0.589**x	0.667	0.445	78.511	<0.001

The coefficient of determination (R^2), that is, the percentage of the variation in the offspring that can be explained by the regression equation, for all floral morphological traits measured vary greatly within and between the two populations studied. In the two populations, the value for coefficient of determination was higher in the Norway population than in the Chiltern population; for example, the coefficient of determination for pistil length in Norway population is R^2 = 87.8%, while in Chiltern population it is R^2 = 68% (Tables 3.18 and 3.19).

Table 3.19 Simple Linear Regressions of offspring floral traits on midparent floral traits in *Collinsia heterophylla* (Chiltern population). Regression equation (*Y* = intercept + slope *x or y* = c + b*x*); slope of regression (bold print) = heritability R^2 = coefficient of determination and regression ANOVA with df = 1, 98

Trait	Regression equation (Slope in bold)	R	R^2	F	P
Corolla length	y=11.843+**0.448**x	0.531	0.282	38.454	<0.001
Wing width	y=8.314+**0.354**x	0.459	0.210	26.117	<0.001
Banner length	y=8.326+**0.028**x	0.042	0.002	0.172	=0.679
Keel length	y=11.662+**0.408**x	0.492	0.242	31.291	<0.001
Stamen1 length	y=6.925+**0.577**x	0.704	0.495	96.212	<0.001
Pistil length	y=2.831+**0.833**x	0.825	0.680	208.417	<0.001
Stamen3 length	y=1.598+**0.880**x	0.870	0.757	305.116	<0.001
Anther-stigma contact	y=0.078+**0 .811**x	0.878	0.772	331.215	<0.001

However, the value of R^2 obtained for wing width and stamen 3 length in the two populations were very similar, Norway population wing width R^2 = 22.1%; stamen3 length R^2 = 75.1% and Chiltern population wing width R^2 = 21%; stamen3 length R^2 = 75.7%. The result obtained showed that the stamens, pistil and anther-stigma contact in the two populations have higher coefficient of determination than the other floral traits measured.

3.4.5 Floral-Traits Heritabilities

Generally, the results obtained for heritabilities show moderate to high narrow-sense heritabilities in the two populations (Tables 3.18 and 3.19). Results obtained from the parent-offspring regressions showed that there are significant heritabilities for all the eight floral traits measured in the Norway populations (0.373 to 0.944, p > 0.001; Tables 3.14). Whereas in the Chiltern population (Table 3.15), seven of the floral traits measured showed significant heritabilities (0.354 to 0.880, p < 0.001); but

heritability of the banner length was not significant (0.028, p = 0.679). The heritability estimates for stamen 1 length is higher in the Norway population (0.791) than in the Chiltern population (0.577). In addition, the pistil length showed higher heritability value in the Norway population (0.944) than in the Chiltern population (0.833). However, heritability values in stamens 3 lengths and ASC were higher in the Chiltern population (0.880 and 0.811) than in the Norway population (0.753 and 0.589). Overall in the two populations, the corolla and keel lengths showed similar heritability values within populations but different between populations (Tables 3.18 and 3.19; Appendix 1.10).

3.5 Discussion

3.5.1 Floral-Traits Variations

The overall distribution of the floral morphological traits variations observed was different from the expected variation. A very large proportion of the floral-traits variations were observed across the five floral developmental stages within plants of *Collinsia heterophylla.* This is similar to the results of Campbell (1992) who observed that in *Ipomopsis aggregata,* definite changes occur from one floral developmental stage to the other. Among-plant variation was not significant while, variation between-population was significant and agrees with the results of most previous studies (e.g. Schwaegerle *et al.*, 1986; Herrera, 1990; Armbruster, 1991 and Dominguez et al., 1998). Also, variations in floral traits observed among parents and offspring populations suggest that, in *Collinsia heterophylla,* the phenotypic variation in floral traits is not only genetic but could be influenced by other environmental factors which need to be further investigated. Williams and Conner (2001) have earlier reported that changes in the environment over a growing season are important factors determining floral trait variation.

3.5.2 Intra-Floral Correlations

The phenotypic correlations among all floral traits (excluding the correlations between banner lengths and wing width in the Chiltern

population) were high in the two populations, ranging from 0.531 to 0.937 (Tables 3.16 and 3.17). These high phenotypic correlations could have two or more possible explanations. The first is that the stamens, pistil and the corolla tube may be more closely related developmentally, thus, the high phenotypic correlation may be due to a high degree of pleiotropy in the genes affecting these traits. Evidence from other plant species show that stamens and petals are more closely related in development than are the remaining floral parts (Hill and Lord, 1989; Hill *et al.*, 1992; Conner and Via, 1993). However, Conner and Via (1993) reported that since phenotypic variation among these same floral traits do not always show a high correlation in other species, the developmental relationship suggested may not be common to all angiosperms. The second possibility for the particularly high phenotypic correlations in floral traits observed could be that selection has acted to increase the correlations for effective pollination (Conner and Via, 1993). Another possibility for these high correlations could be as a result of linkage of genes that control these floral traits development. Strong genetic correlations would restrict independent evolution of floral traits (Caruso, 2006; Bissell and Diggle, 2008).

The results of the genetic correlations for all the traits measured in the two populations were very similar. The low correlations and coefficient of determination observed in the wing width and the banner length in the two populations may be caused by environmental sensitivity. According to Murren (2002), floral trait correlations usually appear plastic, reflecting the environmental sensitivity (see also Schlichting, 1989; Waitt and Levin, 1993). The results obtained indicate that these two floral traits (wing width and banner length) had low repeatabilities and are less likely to respond to selection, but have a high potential for evolutionary modification (Mazer and Dawson, 2001; Parachnowitsch and Elle, 2004). However, because the results of the phenotypic and genetic correlations in the banner length and wing width in one population differ significantly from the other (Tables 3.16 and 3.17), the variations observed could be attributed to some other factors in the environment other than genetic

(maternal effect inclusive). Although, floral traits are often less plastic than vegetative traits (see Williams and Conner, 2001), the results obtained for banner length and wing width indicate some environmental effect (noise). However, stamen1 length, pistil length and stamen3 length are observed to have higher coefficient of determination (linear relationship), suggesting that they would respond more to selection as they seem to have been less affected by environmental noises.

3.5.3 Heritabilities of Floral-Traits

This study has used midparent-regression to estimate heritabilities of the eight floral morphological traits measured in *Collinsia heterophylla*. This is because maternal effects have been reported to be a troublesome source of variation to overcome in plants and animal quantitative-genetic experiments (Falconer and Mackay, 1996). In addition, both theoretical and empirical studies have suggested that maternal influences may slow down or hasten responses to selection and are able to cause large delay in evolutionary responses to selection (Kirkpatrick and Lande, 1989; Wolf, *et al* 1999; reviewed in Hunt and Simmons, 2001). The results obtained in this experiment demonstrate that, for all the eight floral traits measured the phenotypic resemblance between parents and offspring were significant in the Norway population; while in the Chiltern population, seven floral traits showed significant phenotypic resemblance between parents and offspring. While the banner length showed no resemblance between parents and offspring (Tables 3.18 and 3.19). The results obtained in the Norway population are similar to the results obtained for the Norway population of *Collinsia heterophylla* reported by Lankinen *et al.* (2007); for example, heritabilities, correlations and repeatabilities for ASC (h^2 = 0.777, r = 0.667, R^2 = 0.445) obtained in this study are very similar to those observed by Lankinen *et al.* (2007), who reported Pearson correlation - r = 0.668, N = 10, P = 0.035. These heritability results show that floral-traits measured could be said to have been influenced mainly by genetic factors. However, further study of this parent-offspring relationship, using a different breeding design (crosses between more than

one sire to one dam), and relationship between half-sibs are suggested in order to substantiate the result of this experiment.

3.5.4 Evolutionary Interpretations and Consequences of Correlation Patterns

In genetic studies, patterns of genetic correlation may indicate the most likely patterns of future evolution. Therefore, as a result of the high heritabilities and high correlations among most floral morphological traits measured, it is expected that if there is selection for new floral shape or size, the current size (Norway – small and Chiltern – large) is likely to evolve more rapidly. The high genetic correlations between the corolla tube, pistil and the stamens, as well as between the male and female functional parts of the flower, are expected to delay the evolution of different spatial relationships among all the floral traits, except if the high correlations are due to linkage disequilibrium as suggested by Conner and Via (1993).

However, this study was conducted on a small population size; therefore, a definitive interpretation cannot be given at this stage because large populations are suggested to yield more interesting and accurate results in quantitative genetic studies. Nevertheless, the results of the estimates of heritabilities showed some change in genetic variations in floral-traits of *Collinsia heterophylla*. Further estimation of the genetic additive variations in the offspring of a larger population and under different environmental conditions (i.e. field, greenhouse, natural habitat, etc) is required to increase the accuracy of the estimates.

3.6 Conclusion

The results presented in this chapter showed ample phenotypic and genetic variation in all eight floral traits that influence mating systems in *C. heterophylla*. In general, the eight floral morphological traits measured in this study were observed to vary continuously in *Collinsia heterophylla.* Variations in floral morphological traits were observed to be significant within- and between-populations studied; also the eight floral

morphological traits measured vary significantly from one floral developmental stage to the other within- and between- populations. The results presented in this chapter also showed that the variations in most of the floral traits measured are heritable; although some other factors (e.g. maternal and environmental effects) might have influence on some of the traits. Furthermore, floral morphological traits measured were found to be highly correlated suggesting that selection on one trait could result in selection on other traits and consequently, influencing the evolution of mating systems. Given that these floral morphological traits appear to be correlated, it is likely that biotic and/or abiotic environmental conditions influencing one floral trait (e.g. pollination) may have effects on other correlated floral morphological traits. Moreover, the corolla, stamen and pistil lengths were observed to be highly correlated in both study populations, hence it may indicate that any factor that affects one of these traits will likely affect the other two traits. For this reason, the next chapter (chapter four) was designed to investigate the effects of different pollination treatments and consequent fertilisation (leading to the senescence of the pistil) on both the structure and functions of the corolla as well as the stamens.

CHAPTER 4

4.0 Pollination-Induced Flower Senescence in *Collinsia heterophylla*

4.1 Introduction

4.1.1 Flower Longevity

Floral longevity (i.e. the length of time a flower remains open and functional) plays a vital role in the reproductive ecology of plants and has been recently recognised as a trait that could ensure successful pollination in habitats where pollinators are sparse or unreliable (Primack, 1985; Ashman and Schoen, 1994, 1996; Khadari *et al.*, 1995, Rathcke, 2003; Harder and Johnson, 2005; Abdala-Roberts, *et al.*, 2007).

Floral longevity varies significantly among plant species (Primack, 1985). Generally speaking, long life in flowers increases the opportunity for reproductive success through both pollen and ovules, but also requires a high maintenance cost to sustain their functioning and attractiveness to pollinators (Ashman and Schoen, 1997; reviewed in Castro *et al.*, 2008). The size of the floral display and the floral life span as well as its function can influence the total number of pollinator visits (Harder and Johnson, 2005). This consequently affects the quality, and amount of pollen received and exported by the flower, and hence its overall fitness (Primack, 1985; Ashman and Schoen, 1995; Rathcke, 2003; Harder and Johnson, 2005; Castro *et al.*, 2008).

Floral longevity is suggested to be a characteristic adapted to the surrounding ecological conditions such as temperature, water availability, breeding system as well as pollinator visitation rates; therefore, these factors could have a significant effect on the selection for floral longevity (Primack, 1985; Ashman and Schoen, 1994; Yasaka *et al.*, 1998; Sato, 2002; reviewed in Castro *et al.*, 2008). In many angiosperm species, floral longevity can be affected by successful pollination (Devlin and Stephenson, 1984; Richardson and Stephenson, 1989; Proctor and Harder, 1995;

Clayton and Aizen, 1996; Yasaka *et al.*, 1998; reviewed in Sato 2002); for example, it has been reported that plants growing in alpine habitats, which often have few pollinators, have longer floral durations than plants at lower elevations with more abundant, predictable pollinators (see Arroyo *et al.*, 1981; Stratton, 1989; Bingham and Orthner, 1998; Blionis and Vokou, 2001; Blionis *et al.*, 2001; reviewed in Qigang *et al.* 2007).

Although, it is still unexplained what factors are responsible for differences in floral longevity among species with different mating systems (Sato, 2002), selection on flower longevity may result from the trade-off between male- and female-fitness accumulation rates and the costs of floral maintenance or structure (Primack, 1985; Ashman and Schoen, 1994, 1997; Schoen and Ashman, 1995; reviewed in Weber and Goodwillie, 2007). However, models of evolutionary stable strategies (ESS) have been used to explain this variation; this includes both the costs and benefits of outcrossing as well as sustaining a flower (Primack, 1985; Ashman and Schoen, 1994, 1995; Schoen and Ashman, 1995; reviewed in Rathcke, 2003). These models assume that long-lived flowers are selected when either cross-pollination rates or floral maintenance costs are low, while short-lived flowers are selected when cross-pollination rates and maintenance costs are high (Sato 2002). Ashman and Schoen (1994, 1995), observed that this prediction is consistent with the variation in floral longevity in eleven species of ten families studied, although the considerable variation remained unexplained in the species studied.

In pollination studies, much attention has focused upon floral traits that promote successful pollination by increasing the attraction or rewards for animal pollinators or by increasing the effectiveness of pollen transfer (Waser, 1983a & b; Bell, 1985; Real and Rathcke, 1991; Caruso, 2000; Campbell *et al.*, 1996; reviewed in Rathcke, 2003). However, little attention has been given to flower longevity. Nevertheless, from an evolutionary viewpoint, it is proposed that flower longevity in the angiosperms could be shortened after fertilisation to reduce the resource expense and water loss of maintaining open flowers (Stead, 1992; Ashman and Schoen, 1994; reviewed in Weber and Goodwillie, 2007). Although,

species with outcrossing flowers generally last longer than their selfing relatives (Wyatt, 1984; Primack, 1985; Ritland and Ritland, 1989; Dole, 1992), increased intensity of pollination usually shortens flowering time and consequently reduces the cost of floral maintenance. Therefore, whenever cross-pollination is achieved in late-selfing flowers, floral longevity and selfing rates should be decreased by dropping the flower before autonomous self-pollination occurs. Thus, autonomous self-fertilisation will not occur under high pollination intensity (Sato 2002). However in self-compatible hermaphrodite species, when pollinators are limited, reproductive assurance is achieved through autonomous self pollination after a flower has been receptive to outcross pollen for several days (Motten, 1982; Piper *et al.*, 1986; Lloyd and Schoen, 1992; Rathcke and Real, 1993; Kalisz *et al.*, 1999; reviewed in Sato, 2002). As a result, the timing of autonomous self-fertilisation will certainly influence both the selfing rate and floral longevity (Sato, 2002).

The effects of pollination on variation in floral longevity have been assessed in several plant species and, in general, the results have revealed a decrease in floral longevity with male and/or female accrual rates, i.e. with the accomplishment of flower function (Stead and Moore, 1979; Ishii and Sakai, 2002; Stpiczynska, 2003; Abdala-Roberts *et al.*, 2007; reviewed in Castro *et al.*, 2008). According to Porat *et al.* (1994), if floral longevity can react to pollen distribution and/or pollen reception, then it could have the necessary flexibility for the balance between reproductive output and resource efforts, for example, when pollinators are irregular or random (Abdala-Roberts *et al.*, 2007). Therefore, the selection of a longer floral life span could be attributed to scarce pollinators when floral maintenance costs are low (Ashman and Schoen, 1994; reviewed in Castro *et al.*, 2008).

4.1.2 Flower Senescence

According to Rogers (2006), species-specific flower senescence occurs as a way of reducing the expenses of maintaining the flower beyond its useful lifespan; this is because the flower can be a considerable sink on plant resources. Although flowers have a species-specific, limited life span with an irreversible programme of senescence (Rogers, 2006), flower senescence is normally triggered by fertilisation in many angiosperms (Gori, 1983; Stead, 1992; Yasaka *et al.*, 1998; Underwood *et al.*, 2005). However, in some species it is a process mediated by plant growth regulators (PGR) - ethylene (Stead, 1992; O'Neill, 1997; VanDoorn, 2002; Rogers, 2006; reviewed in Weber and Goodwillie, 2007) and therefore, largely independent of biotic and abiotic environmental factors (Roger, 2006). Nevertheless, in species where pollination is the trigger, the effect of pollination is usually mediated by the plant growth regulator (PGR) ethylene. In some species ethylene is a major regulator of floral senescence, but in other species it plays a very minor role and the co-ordinating signals involved remain un-described. Other PGRs such as cytokinin and brassinosteroids are also important but their role is understood only in some specific systems (Rogers, 2006).

Recent studies have suggested that the rate of corolla senescence can vary within populations. This indicates the importance of incorporating conditional responses in fitness accrual rates into existing floral longevity model (Sargent and Roitberg, 2000; Evanhoe and Galloway, 2002; Rathcke, 2003). However, what is still uncertain is whether such conditional responses vary among natural populations that experience differences in fitness accrual rates and floral maintenance costs. In the case of hermaphrodite flowers, further consideration is given to whether the functional gender phases (i.e., staminate, pistillate) respond differently to variation in fitness-accrual rates (Ashman, 2004; reviewed in Giblin, 2005).

It has been suggested that the rates of fitness accrual through ovule fertilisation and pollen dispersal can be distinguished by autonomous selfing; therefore, fertilisation-induced flower senescence may have

possible consequences for mating-system evolution (Weber and Goodwillie, 2007). However, in obligate outcrossing hermaphrodite species, where fertilisation is entirely pollinator-mediated, and both male and female fitness depend on the action of the same pollen vector. Therefore, fitness through ovule fertilization and pollen dispersal must be correlated to some extent, although they may accrue at different rates (Ashman and Schoen, 1994; Schoen and Ashman, 1995; reviewed in Weber and Goodwillie, 2007). Consequently, in an outcrossing species, ovule fertilisation is expected to trigger flower senescence only after substantial pollen grains have been dispersed. Similarly, autonomous self-fertilisation of ovules that occurs early in anthesis may trigger flower senescence before any opportunity for dispersal of pollen by a vector. Therefore, selfing-induced flower senescence might be viewed as a potential source of pollen discounting, which is defined as a reduction in male outcross success that results from self-fertilisation (Holsinger *et al.*, 1984; Weber and Goodwillie, 2007).

However, Sato (2002) reported that the effect of self-fertilisation (selfing) on flower senescence has received little attention. Indeed, the selective role of this effect in the evolution of the selfing rate has hardly ever been measured (see details in Weber and Goodwillie, 2007). The consequences of selfing-induced flower senescence will depend on the pollination context. For example, whenever pollen dispersal is minimal, or pollinator visitation is low, floral longevity will normally have little or no consequence for male fitness. Therefore, selfing-induced flower senescence may limit opportunities for male success through pollen dispersal (Weber and Goodwillie, 2007). Thus, one important aspect of this study is to examine the potential cost of autonomous selfing in different populations of *Collinsia heterophylla.*

The study plant, *Collinsia heterophylla* (Chinese houses), is an ideal system for the study of the effect of pollination treatments on flower longevity; it is a self-compatible annual herb which exhibits a mixed mating system. Flower size and the timing of self-fertilisation vary within and among species of *Collinsia* (Armbruster *et al.*, 2002). In most species,

irrespective of the flower size, the timing of stigma receptivity prevents early self-fertilisation.

Although, many studies have shown that pollinators decrease floral longevity in orchids (Proctor and Harder, 1995; Clayton and Aizen, 1996; Van Doorn, 1997; Martini et al., 2003; Stpiczynska, 2003), much less attention has been paid to the relationship between reproductive costs (e.g. flower construction, maintenance) and floral longevity (Abdala-Roberts *et al.*, 2007). Therefore, in order to broaden our understanding of the relationship between flower construction (e.g. size) and flower longevity, *C. heterophylla* was used as a model plant. Also, because the evolutionary forces that are responsible for the variation in floral longevity among populations are yet to be well understood, this study seeks to assess the effect of four different pollination treatments (crossed - HC; selfed – HS; bagged – BG; and emasculated – EM) on flower longevity in the two populations of *C. heterophylla*, and assess its implication for mating system evolution. In this study attempt is made to provide answers to the following questions with the aim of increasing our understanding of the subject in this chapter. The questions are as follows:

(i) Does the timing of fertilization affect the rate of flower senescence?
(ii) Does type of pollination (self or outcross) affect the rate of flower senescence?
(iii) Does flower longevity differ among populations under the same pollination treatments and environmental factors?
(iv) Does emasculation increase floral longevity?

This study therefore, investigates the changes in floral longevity following four different pollination treatments under natural conditions in the greenhouse in the absence of pollinators. The study discusses the possible consequences of autonomous self-pollination on variation in floral longevity. In addition, the effect of floral developmental stages at pollination and the possible consequences for reproductive vigour are examined.

4.2 Materials and Methods

4.2.1 Study Species

The study plant *Collinsia heterophylla* Buist (Plantaginaceae) is easy to cultivate, as the seeds germinate approximately two weeks after sowing. The plant begins to flower at about 10 weeks after planting. Therefore, one generation cycle takes about 4 months. *Collinsia heterophylla* flowers between March and June depending on latitude and elevation. The flowers are held out on the branch by short stalks and arranged in whorls on spikes, calyx lobes generally acute, glabrous to shaggy; corolla 10-24mm (depending on the flower size) and usually glabrous outside whereas the throat is hairy inside (see detailed description of the *C. heterophylla* flower in chapter three, section 3.2.1).

4.2.2 Flower Development and Longevity

Collinsia heterophylla starts flower initiation at approximately 6 – 10 weeks after planting, and flower primordial last between 11 – 25 days (depending on species and prevailing environmental conditions) before flower opens. Open pollinated flowers of *Collinsia heterophylla* live for approximately 1 to 5 days before senescing (pers. obs.). Even though the corolla is securely attached to the receptacle at the time of pollination, the attachment soon becomes weak, within 4-6hrs after pollination; this makes it possible for the corolla to be detached easily by a slight tug on the lobes (Schrock and Palser, 1967).

If undisturbed, a corolla withers and become detached within 24hr after pollination and the style also withers within 24hr, if the stigma is in the receptive condition when dusted with pollen. However, if the stigma is not receptive at the time of pollination or there is no pollination, the corolla does not wither for several days and the style does not wither until 2 weeks after the time when pollination should have occurred (Schrock and Palser, 1967 cited by Bello *et al.*, 2004 and Lankinen *et al.*, 2009).

4.2.3 Sampling

The seeds used for this experiment were collected from two different populations. One population was generated from seeds of inbred lines obtained from Norway but originally from America. The second population was collected from an open-pollinated population of *Collinsia heterophylla* growing in the open field at Chiltern Seeds, Ulverston, Cumbria, UK, and subjected to two generations of self-fertilization. The two populations were grown during the spring/summer of 2007 in the greenhouse of the University of Portsmouth, UK.

Table 4.1: Sample sizes of *Collinsia heterophylla* populations used to estimate flower senescence, population 1= seeds obtained from Chiltern seeds UK; population 2= seeds obtained from Norway originally from California (USA).

Population	Number of plants
1. Chiltern	30
2. Norway/American	30
Total	60

4.2.4 Experimental Site

This study was carried out in the newly constructed greenhouse of the University of Portsmouth UK during the spring/summer of 2007. Plants were germinated and grown in multi-purpose compost in the well protected greenhouse. Plants were transplanted at about three weeks after germination, when the second pair of leaves had emerged. Each plant was grown in a separate flower pot to maturity. Approximately 400 plants were grown for the purpose of this experiment; each population had about 200 plants, placed in a randomised complete block (RCB) design. The pollination treatment experiment was conducted in the glasshouse next to the well protected greenhouse. This is to provide opportunities for open pollination by insect pollinators. So, 100 randomly selected plants from each of the two populations were grown in separate flower pots in the

glasshouse. The plants were regularly watered and left to grow till flower primordial appeared and subsequently flowers opened. The two populations studied in this experiment are labelled as Chiltern population and Norway population. In this chapter, the Chiltern population was named population 1 and the population obtained from Norway was named population 2 for the purpose of this experiment.

4.2.5 Pollination Protocol

To assess the effect of pollen addition and/or removal on flower longevity, pollination experiments were designed to investigate the effect of the four main pollination treatments on floral longevity, and the rate of flower senescence under four different stages of floral development (i.e. stages 0 – 3). The pollination experiment was carried out as follows: during spring/summer of 2007, four pollination treatments were applied to carefully selected flowers in the first four stages of flower development (i.e. stages 0 – 3, numbers represent number of dehisced anthers; see Armbruster *et al.*, 2002). The pollination treatments applied to the two populations are as follow: (i) crossed (HC); (ii) bagged (BG); (iii) selfed (HS); and (iv) emasculated (EM). A fifth group was added to the pollination treatments; this is the unmanipulated (UM) treatment, they were open-pollinated flowers (i.e. not bagged and no treatment applied - serving as the control).

The pollination treatments described were replicated on thirty individuals randomly chosen from each of the two populations (i.e. 2 x 30 = 60 plants in total). A set of all four treatments was repeated on three flowers at each of the four stages of floral development on each of the thirty randomly sampled plants (3 flowers x 4 floral stages x 4 treatments x 30 plants = 1440/population and a total of 2880 crosses in the two populations). Individuals from the same population were pollinated on the same day to reduce within-population errors; therefore, 1440 crosses were completed each day (population 1 – Chiltern population on the 16/05/2007 and population 2 – Norway on the 17/05/2007). To achieve pollination in all hand-pollination treatments, flowers were carefully

emasculated at stages 0, 1, 2, and 3 to prevent the deposition of self-pollen on the stigma. Afterwards, pollen from two newly dehisced anthers (self – and cross –pollen) were dusted on a glass slide and directly applied to the stigma of each flower until the stigma was fully covered with pollen. All four treatments were bagged with empty tea bags to prevent ingress by external pollen grains or contamination of any type. The bagged treatments were properly labelled with tags having the stage of floral development and the treatment applied; this was to ensure the appropriate label was used in order to make it easy to identify each treatment and the floral developmental stage. The flowers were observed at noon each day after treatments were applied.

4.2.6 Observations and Data Collection

A flower was considered senesced when petals were closed or the flower was no longer upright and therefore unavailable for pollination. The longevity of each flower was observed and recorded in increments of days after treatments were applied. Mean floral longevity for each treatment at each floral developmental stage was calculated by finding the average number of days to senescence for the three flowers measured at each developmental stage per pollination treatment and the mean was recorded in number of days. The same data was collected and mean calculated for each of the 60 plants observed. The results of the pollination treatments: [crossed (HC), bagged (BG), selfed (HS), emasculated (EM) and unmanipulated (UM)] were computed and compared to assess if pollen deposit and/or removal affect flower longevity. The selfed (HS) treatment controlled for factors that prevent self-pollination, such as stigma-anther separation, while the bagged and un-manipulated flowers relied on autonomous self-fertilization.

4.2.7 Statistical Methods and Analysis

All statistical analyses were completed using the program SPSS 16.0 (SPSS, 2008). The data collected were organised, summarised and described using descriptive statistics. The descriptive statistics were

computed for flower longevity on a pooled data for treatments in the two populations studied; and these were presented as mean, standard deviation, variance, standard error of the mean, minimum and maximum values. Also a separate descriptive statistics was computed to illustrate clearly the median, standard error of mean, range, skewness and geographic mean of each of the two populations. The pollination treatments were represented by letters for easy representation; (i) crossed (HC); (ii) bagged (BG); (iii) selfed (HS); and (iv) emasculated (EM). The open pollinated flowers were referred to as the un-manipulated (UM) flowers. The two populations used in this investigation were represented by numbers; the Chiltern population is labelled Population 1 and the Norway population is labelled Population 2. The floral stages were represented as stage 0 for day one through to stage 3 as day four of anthesis. The variable (flower longevity) was measured in number of days to flower senescence.

To assess the effect of pollen addition and/or pollen removal on floral longevity in the two populations studied, the descriptive statistics was used to compare the means and standard deviations. Prior to further statistical analyses, the data collected were tested for normality and homogeneity of variance, as described in chapter three, section 3.3. The data did not conform to the criteria for parametric test (Appendix 2.0); therefore, the variations in flower longevity were assessed for significance using the non-parametric statistics on SPSS 16.0 (SPSS, 2008). Consequently, to examine if the difference in mean number of days to flower senescence observed at each floral developmental stages under the four pollination treatments in the two populations was significantly different, Mann-Whitney U test for two-independent samples is computed using SPSS 16.0 (SPSS, 2008). The grouping variables was the populations (1 = Chiltern and 2 = Norway) and the test variable was the number of days to flower senescence.

To investigate if number of days to flower senescence was significantly different under the four main population treatments (HS, HC, BG, EM, and UM), the Kruskal-Wallis H several independent samples test

was used. The grouping variable was the treatment while the test variable was the number of days to senescence. Similarly, to assess if the variation in number of days to flower senescence under the different treatments at each floral developmental stage was significantly different, the Kruskal-Wallis H several independent sample test was computed using SPSS 16.0 (SPSS, 2008). The grouping variable was the floral developmental stage while the test variable was the individual pollination treatments (i.e. HS, HC, BG, EM, and UM). Since the results obtained showed that flower longevity in a pooled data for the two populations was significantly different for all pollination treatments under the four developmental stages, each of the two populations was examined independently.

To assess the data collected for differences within each population, the Norway and the Chiltern populations were examined independently. The effects of the pollination treatments on flower longevity across floral developmental stages within each of the two populations studied was examined using the Kruskal-Wallis H several independent samples test computed for each population. The grouping variable was the floral developmental stages and the individual treatments were the test variables. The results obtained showed that there were significant differences among treatments across the four floral developmental stages; therefore, a *Post hoc* Dunnett T3 test for unequal variances was computed for each population to find out where the differences laid.

In addition to the tests above, flower longevity under autonomous selfing was assessed in the two populations across floral developmental stages; data were pooled from both populations for bagged treatment (BG). The Mann-Whitney U test for two independent samples was used to compare the bagged treatment between the two populations (using population as the grouping variable and the bagged pollination treatment as the test variables); while the Kruskal-Wallis H test for several independent samples was used to compare the bagged treatment among the four floral developmental stages (the grouping variable). All tests were performed using SPSS 16.0 (SPSS, 2008).

4.3 Results

4.3.1 Variation in Mean Number of Days to Flower Senescence Betweeen-Populations of *Collinsia heterophylla* Under Different Pollination Treatments

The mean number of days to flower senescence (from the pooled data for the two populations) under the four pollination treatments (including the control - unmanipulated) vary considerable between the two populations of *C. heterophylla* studied (Table 4.2).

Table 4.2: Descriptive Statistics of population treatments in *Collinsia heterophylla*; variables showing Mean number of days to flower senescence, Standard deviation (SD), Variance (*V*), Standard Error of mean ($S.E_M$), Min. = minimum days to senescence, Max. = maximum days to senescence); treatments (HC = crossed, BG = bagged, HS = selfed, EM = emasculated, UM = unmanipulated). The variables are measured in number of days to senescence.

Treatment	*Population*	*Mean*	*SD*	*V*	$S.E_M$	*Min.*	*Max.*
Crossed (HC)	**Chiltern**	4.84	1.091	1.190	0.100	3.00	7.00
	Norway	2.84	1.052	1.106	0.096	1.00	5.33
Bagged (BG)	**Chiltern**	7.76	1.146	1.313	0.105	4.33	9.67
	Norway	4.22	1.472	2.166	0.134	2.00	7.33
Selfed (HS)	**Chiltern**	4.87	1.307	1.707	0.119	3.00	8.33
	Norway	3.04	0.925	0.856	0.085	1.67	5.33
Emasculated (EM)	**Chiltern**	9.15	1.511	2.282	0.138	5.67	11.67
	Norway	9.24	0.815	0.665	0.074	7.67	11.33
Unmanipulated (UM)	**Chiltern**	4.98	0.448	0.201	0.041	4.00	5.67
	Norway	2.70	0.452	0.204	0.041	1.67	3.67

Generally, irrespective of floral developmental stages, the Chiltern population shows higher mean number of days to flower senescence than the Norway population for all four pollination treatments. But in the emasculated (EM) pollination treatment the mean number of days to flower senescence is slightly higher in the Norway population (9.24 days)

than in the Chiltern population (9.15 days); even though the difference is only marginal (Table 4.2).

Table 4.3: Descriptive Statistics of Pollination Treatments across the four floral developmental stages (stages 0 – 3) on a pooled data for the two populations (Chiltern and Norway) of *Collinsia heterophylla*; variables (Mean number of days to flower senescence, SD = Standard deviation, V = Variance, SE = Standard Error of mean); treatments (HC = crossed, BG = bagged, HS = selfed, EM = emasculated, UM = unmanipulated). The variables are measured in number of days to senescence.

Treatment	Statistics	Stage 0	Stage 1	Stage 2	Stage 3
HC	**Mean**	4.14	4.42	3.76	3.10
	SD	0.689	1.179	1.794	1.575
	S.E	0.089	0.152	0.232	0.203
	V	0.475	1.390	3.219	2.481
HS	**Mean**	4.08	4.25	4.16	3.31
	SD	0.753	0.983	2.117	1.413
	S.E	0.097	0.127	0.273	0.182
	V	0.567	0.967	4.480	1.997
BG	**Mean**	7.76	5.88	5.35	4.98
	SD	1.337	1.923	2.213	2.177
	S.E	0.173	0.248	0.286	0.281
	V	1.789	3.699	4.896	4.741
EM	**Mean**	9.92	9.98	8.57	8.31
	SD	0.698	0.583	1.210	1.133
	S.E	0.090	0.075	0.156	0.146
	V	0.488	0.340	1.465	1.284
UM	**Mean**	3.84	3.84	3.84	3.84
	SD	1.235	1.235	1.235	1.235
	S.E	0.159	0.159	0.159	0.159
	V	1.524	1.524	1.524	1.524

4.3.2 Variation in Mean Number of Days to Flower Senescence Across Floral Developmental Stages Under Different Pollination Treatments

The four pollination treatments (HC, BG, HS, EM) show a consistent pattern of variation in the mean number of days to flower senescence on a pooled data for the two populations. Under the four pollination treatments the mean number of days to flower senescence decreased gradually across the floral developmental stages, that is, from stage 0 to stage 3 (see Table 4.3). But in the crossed (HC), selfed (HS) and emasculated (EM) pollination

treatments, the pattern observed is interesting as the mean number of days to senescence at 'stage 1' in the three pollination treatments increased slightly than at 'stage 0' and then decreased at 'stage 2'. Overall the emasculated treatment show the highest number of days to flower senescence at all the four floral developmental stages. The least number of days to flower senescence is observed at floral developmental stage 3 of the crossed (HC).

To examine if the variations observed among populations and across floral developmental stages is significantly different, the Mann-Whitney U test for two independent samples shows that the mean rank in the Chiltern population is higher than in the Norway population (758.33 in Chiltern and 442.67 in Norway). The test statistics shows that there is significant difference in the mean number of days to flower senescence in the two populations of *C. heterophylla* studied (Mann-Whitney U test: U = 8530.0, n_1 = 600, n_2 = 600, P < 0.001) (Table 4.4 and 4.5).

Table 4.4 Mann-Whitney U test showing the mean ranks of the number of days to flower senescence for the two populations of *C. heterophylla* (Chiltern and Norway, using population as the grouping variable and number of days to senescence as the test variable; the degree of freedom = 1 and N represents the number of measurements.

	Population	**N**	**Mean Rank**	**Sum of Ranks**
Days to Senescence	Chiltern	600	758.33	455000.00
	Norway	600	442.67	265600.00
	Total	1200		

Table 4.5 Mann-Whitney U test showing the Test Statistics (X^2) of the number of days to flower senescence for the two populations of *C. heterophylla* (Chiltern and Norway), using population as the grouping variable and number of days to senescence as the test variable; the degree of freedom = 1 and N represents the number of measurements.

	Days to senescence
Mann-Whitney U	85300.000
Wilcoxon W	265600.000
Z	-15.793
P	<0.001

The variation in number of days to flower senescence is also significantly different under the four pollination treatments across the four floral developmental stages. The Kruskal-Wallis H test shows that the emasculated (EM) flower has the largest mean rank = 814.45, while the crossed (HC) = 295.45 has the smallest (Table 4.6). The test statistics (Table 4.7) shows that this difference in mean rank across treatments is significant (X^2 = 554.720; df = 3; p < 0.001).

Table 4.6 Kruskal-Wallis H test showing the mean ranks of number of days to flower senescence for the four pollination treatments (HC, BG, HS, EM) in a pooled data of the two populations of *C. heterophylla* (Chiltern and Norway); using pollination treatments as the grouping variable and number of days to senescence as the test variable. The df = 3, N represents the number of measurements.

	Treatments	N	Mean Rank
Days to Senescence	Bagged (BG)	240	507.34
	Selfed (HS)	240	304.72
	Crossed (HC)	240	295.49
	Emasculated (EM)	240	814.45
	Total	960	

Table 4.7 Kruskal-Wallis H test showing the Test Statistics (X^2) of number of days to flower senescence for the four pollination treatments (HC, BG, HS, EM) in a pooled data of the two populations of *C. heterophylla* (Chiltern and Norway); using pollination treatments as the grouping variable and number of days to senescence as the test variable. The df = 3, N represents the number of measurements.

	Days to Senescence
Chi-Square	554.720
df	3
P	<0.001

The results obtained from Kruskal-Wallis H test also reveals that even under the same pollination treatment, flowers at different floral developmental stages senesced at different times (number of days; Table 4.8). The results of Kruskal-Wallis H test shows mean rank for flowers under crossed (HC) pollination treatment to be largest at stage 1 = 143.00

and smallest at stage 3 = 85.56. In the bagged (BG) pollination treatment mean rank at stage 0 = 172.05 is largest and smallest at stage 3 = 86.78.

Table 4.8 Kruskal-Wallis H test showing the mean ranks of number of the floral developmental stages (stages 0 to 3) for the four pollination treatments (HC, BG, HS, EM) in a pooled data of the two populations of *C. heterophylla* (Chiltern and Norway); using floral developmental stages the grouping variable and pollination treatments as the test variable. The df = 3, N represents the number of measurements.

	Floral Stages	**N**	**Mean Rank**
Crossed (HC)	stage 0	60	137.04
	stage 1	60	143.00
	stage 2	60	116.40
	stage 3	60	85.56
	Total	240	
Bagged (BG)	stage 0	60	172.05
	stage 1	60	120.82
	stage 2	60	102.35
	stage 3	60	86.78
	Total	240	
Selfed (HS)	stage 0	60	134.64
	stage 1	60	141.32
	stage 2	60	117.82
	stage 3	60	88.22
	Total	240	
Emasculated (EM)	stage 0	60	162.84
	stage 1	60	170.18
	stage 2	60	82.70
	stage 3	60	66.28
	Total	240	

The result of the selfed (HS) pollination treatment shows a similar pattern to the crossed pollination treatment. The emasculated (EM) pollination treatment shows that the mean rank is largest at stage 1 = 170.18 and smallest at stage 3 = 66.28 (Table 4.8). The result of the computed Kruskal-Wallis H test statistics (X^2) with df = 3 shows that the variation in number of days to flower senescence observed at different

floral developmental stages under the four pollination treatments (X^2 crossed, bagged, selfed, emasculated, P < 0.001; Table 4.9).

Table 4.9 Kruskal-Wallis H test showing the Test Statistics (X^2) of number of the floral developmental stages (stages 0 to 3) for the four pollination treatments (HC, BG, HS, EM) in a pooled data of the two populations of *C. heterophylla* (Chiltern and Norway); using floral developmental stages the grouping variable and pollination treatments as the test variable. The df = 3, N represents the number of measurements.

	Crossed (HC)	Bagged (BG)	Selfed (HS)	Emasculated (EM)
Chi-Square	25.310	51.518	21.080	108.849
df	3	3	3	3
P	<0.001	<0.001	<0.001	<0.001

4.3.4 Effect of Pollination Treatments on Flower Longevity Across Floral Developmental Stages Between Chiltern and Norway Populations

In the Norway population (Tables 4.10 and 4.11) the results show a regular pattern in number of days to flower senescence across floral developmental stage for the four pollination treatments. Flowers at stage 0 generally lived longer after pollination treatments [mean ± s.d; HC = 3.90±0.35, BG = 6.49±0.47, HS = 3.78±0.46, but emasculated (EM) flowers at stage 1 lived much longer (9.87±0.61) than at stage 0 (9.72±0.48)]; while floral longevity decreased with flower age (floral developmental stages 0 to 3) in all pollination treatments (see Tables 4.10 and 4.11).

In contrast to the Norway population, the Chiltern population shows an interesting pattern across all floral developmental stages. The crossed (HC) and selfed (HS) show higher mean number of days to flower senescence at stages 1 and 2 than at stage 0. Both treatments (HC and HS) show a gradual rise in flower longevity from stage 0 through stage 1 and reaching the highest at stage 2 (mean ± s.d number of days to flower senescence: stage 0 - HC = 4.39±0.85, HS = 4.39±0.87; stage 1 – HC = 5.14±1.23, HS = 4.63±1.10; stage 2 – HC = 5.36±1.06, HS = 5.98±1.45); then show a rapid drop at stage 3 (mean ± s.d: HC = 4.46±0.90, HS =

4.47±1.09) (Table 4.10 and Table 4.11). However the bagged (BG) and emasculated (EM) pollination treatments show a pattern similar to that observed in the Norway population (i.e. decrease in flower longevity from stage 0 to stage 3 (mean ± s.d: BG - stage 0 = 9.02±0.31, stage 1 = 7.65±0.86, stage 2 = 7.35±1.14, stage 1 = 7.02±0.92; EM - stage 0 = 10.12±0.82, stage 1 = 10.10±0.54, stage 2 = 8.22±1.51, stage 1 = 8.14±1.50).

Table 4.10 Descriptive statistics of Chiltern and Norway population mean, standard error of mean, range, standard deviation, variance, skewness and geographic mean for the four treatments (Crossed (HC), Bagged, Hand-selfed, emasculated and unmanipulated) at floral developmental stages 0 and 1. The variables are measured in number of days to flower senescence.

Floral Stage	Population	Statistics	Crossed (HC)	Bagged (BG)	Selfed (HS)	Emasculated (EM)	Unmanipulated (UM)
Stage 0	**Chiltern**	Mean	4.39	9.02	4.39	10.12	4.98
		S.E. of Mean	0.155	0.057	0.158	0.150	0.083
		Range	2.67	1.34	2.67	2.67	1.67
		St. Deviation	0.849	0.313	0.867	0.824	0.454
		Variance	0.720	0.098	0.751	0.679	0.206
		Skewness	0.273	-0.140	-0.448	0.004	-0.134
	Norway						
		Mean	3.90	6.49	3.78	9.72	2.70
		S.E. of Mean	0.064	0.086	0.083	0.088	0.084
		Range	1.34	1.66	1.33	1.67	2.00
		St. Deviation	0.352	0.469	0.457	0.481	0.458
		Variance	0.124	0.220	0.209	0.231	0.210
		Skewness	-0.109	0.041	-0.404	0.059	-0.105
Stage 1	**Chiltern**						
		Mean	5.14	7.65	4.63	10.10	4.98
		S.E. of Mean	0.224	0.157	0.201	0.099	0.083
		Range	4.00	3.00	3.67	2.34	1.67
		St. Deviation	1.225	0.859	1.102	0.540	0.454
		Variance	1.501	0.738	1.214	0.292	0.206
		Skewness	-0.482	0.840	0.106	1.505	-0.134
	Norway						
		Mean	3.70	4.11	3.87	9.87	2.70
		S.E. of Mean	0.091	0.099	0.122	0.111	0.084
		Range	2.33	2.00	2.66	2.66	2.00
		St. Deviation	0.498	0.542	0.670	0.610	0.458
		Variance	0.248	0.294	0.449	0.372	0.210
		Skewness	1.271	0.392	0.050	0.206	-0.105

Table 4.11 Descriptive statistics of Chiltern and Norway population mean, standard error of mean, range, standard deviation, variance and skewness for the four treatments (Hand-crossed, Bagged, Hand-selfed, emasculated and unmanipulated) at floral developmental stages 2 and 3. The variables are measured in number of days to flower senescence.

Floral Stage	Population	Statistics	Crossed (HC)	Bagged (BG)	Selfed (HS)	Emasculated (EM)	Unmanipulated (UM)
Stage 2	**Chiltern**						
		Mean	5.36	7.35	5.98	8.22	4.98
		S.E. of Mean	0.193	0.208	0.266	0.276	0.082
		Range	3.33	4.34	4.66	5.33	1.67
		St. Deviation	1.057	1.142	1.455	1.511	0.454
		Variance	1.118	1.304	2.117	2.283	0.206
		Skewness	-0.236	-1.293	-0.311	-0.110	-0.134
	Norway						
		Mean	2.16	3.36	2.34	8.91	2.70
		S.E. of Mean	0.067	0.116	0.074	0.123	0.084
		Range	1.33	3.00	2.00	2.33	2.00
		St. Deviation	0.368	0.637	0.406	0.672	0.458
		Variance	0.135	0.405	0.165	0.451	0.210
		Skewness	0.273	0.569	1.053	0.042	-0.105
Stage 3	**Chiltern**						
		Mean	4.46	7.02	4.47	8.14	4.98
		S.E. of Mean	0.164	0.168	0.199	0.275	0.083
		Range	4.00	4.00	4.00	5.00	1.67
		St. Deviation	0.898	0.922	1.089	1.505	0.454
		Variance	0.807	0.850	1.186	2.264	0.206
		Skewness	0.835	-0.428	0.724	-0.314	-0.134
	Norway						
		Mean	1.62	2.93	2.16	8.47	2.70
		S.E. of Mean	0.055	0.070	0.061	0.099	0.084
		Range	1.33	1.67	1.00	2.00	2.00
		St. Deviation	0.301	0.385	0.335	0.543	0.458
		Variance	0.090	0.148	0.112	0.295	0.210
		Skewness	-0.050	0.411	0.012	0.477	-0.105

On the whole, the emasculated (EM) flowers across floral developmental stages 0 to 3 generally lived longer in the two populations (Chiltern and Norway), than the selfed (HS), crossed (HC), bagged (BG) and the control (unmanipulated – UM). Furthermore the results obtained show that the mean number of days to flower senescence in the unmanipulated (UM) treatment (i.e. the control) vary between the two populations studied; with the Chiltern population having a higher mean than the Norway population (UM – Chiltern = 4.98±0.45 > UM – Norway = 2.70±0.46) [Table 4.10 and 4.11].

4.3.5 Effect of Pollination Treatments on Flower Longevity Across Floral Developmental Stages Within the Chiltern and the Norway Populations

To examine if these variations in means observed in Tables 4.10 and 4.11 are significantly different, a Kruskal-Wallis H test for several independent samples (using floral developmental stages as the grouping variable and number of days to flower senescence as the test variable) is used to analyse the data. The results obtained show that in the Chiltern population the flowers at stage 2 lived longer than flowers at stages 0, 1, and 3 (Table 4.12).

Table 4.12 Kruskal-Wallis H test showing the mean ranks for floral developmental stages, (stages 0 to 3) in the Chiltern population of *C. heterophylla,* using the floral developmental stages as the grouping variable and number of days to senescence as the test variable; the df = 3, N represents the number of measurements.

	Floral Stages	N	Mean Rank
Days to senescence	stage 0	30	46.60
	stage 1	30	69.60
	stage 2	30	78.22
	stage 3	30	47.58
	Total	120	

Table 4.13 Kruskal-Wallis H test showing the test statistics (X^2) for the floral developmental stages (Stages 0 to 3) in the Chiltern population of *C. heterophylla,* using floral developmental stages as the grouping variable and number of days to flower senescence as the test variable; the df = 3.

	Days to Senescence
Chi-Square	19.003
df	3
P	<0.001

However, in the Norway population the flowers at stage 0 lived longer than at stages 1, 2, and 3 (Table 4.14). The Kruskal-Wallis H test statistics obtained for the number of days to flower senescence in the two populations studied reveals that the variation in mean ranks observed at each of the floral developmental stages (stages 0 to 3) within each population is statistically significant at 0.05 level [X^2 - Chiltern = 19.003, df = 3, P < 0.001 (Table 4.13); X^2 – Norway = 97.585, df = 3, P < 0.001 (Table 4.15)].

Table 4.14 Kruskal-Wallis H test showing the mean ranks for floral developmental stages (Stages 0 to 3) in the Norway population of *C. heterophylla,* using floral developmental stages as the grouping variable and number of days to senescence as the test variable; the df = 3, N represents the number of measurements.

	Floral Stages	N	Mean Rank
Days to Senescence	stage0	30	95.27
	stage1	30	85.70
	stage2	30	41.28
	stage3	30	19.75
	Total	120	

Table 4.15 Kruskal-Wallis H test showing the test statistics (X^2)for the floral developmental stages (Stages 0 to 3) in the Norway population of *C. heterophylla,* using floral developmental stages as the grouping variable and number of days to flower senescence as the test variable; the df = 3.

	Days to Senescence
Chi-Square	97.585
df	3
P	<0.001

Furthermore, to examine how the pollination treatments affect the variations in mean ranks observed at the floral developmental stages in Tables 4.12 and 4.14 above, a Kruskal-Wallis H test for several independent samples (using floral developmental stages 0 to 3 as the grouping variable and each pollination treatment as the test variables) is

used to analyse the data. The mean rank results obtained show that in the Chiltern population, the crossed (HC) and selfed (HS) pollination treatments show similar results as the flowers at developmental stage 2 lived longer than flowers at stages 0, 1 and 3 (see Table 4.16). In the bagged (BG) pollination treatment, the flowers at stage 0 lived longer than flowers at stages 1, 2, and 3 (BG – stage 0 = 101.17, stage 1 = 54.35, stage 2 = 49.23, stage 3 = 37.25). The emasculated (EM) pollination treatment shows that the flowers at stage 1 lived longer than at stages 0, 2 and 3 (EM – stage 0 = 82.88, stage 1 = 83.55, stage 2 = 39.28, stage 3 36.28) [Table 4.16]. In addition, the Kruskal-Wallis test statistics computed confirms that the variation in mean ranks observed for all floral developmental stages under the four pollination treatments are significantly different at df = 3 (X^2 – HC = 19.003, P < 0.001; X^2 – BG = 59.052, P < 0.001; X^2 – HS = 21.095, P < 0.001; X^2 – EM = 51.870, P < 0.001) [Table 4.17].

The mean rank results obtained in the Norway population, reveals that the crossed (HC), and bagged (BG) pollination treatments follow a similar pattern; with the flowers at stage 0 having the highest number of days to flower senescence and the flowers at stage 3 having the least number of days to senescence (HC – stage 0 = 95.27, stage 1 = 85.70, stage 2 = 41.28, stage 3 = 19.75; BG - stage 0 = 105.50, stage 1 = 69.35, stage 2 = 42.17, stage 3 = 20.98) [Table 4.18]. In contrast, the results observed in the selfed (HS) and emasculated (EM) pollination treatments are similar to each other, where the flowers at stage 1 lived longer than flowers at stage 0, 2, and 3 (HS – stage 0 = 88.78, stage 1 = 91.07, stage 2 = 35.32, stage 3 = 26.85; EM - stage 0 = 81.60, stage 1 = 86.55, stage 2 = 46.15, stage 3 = 27.70) [Table 4.18].

Table 4.16 Kruskal-Wallis H test showing the mean ranks of pollination treatments (HC, HS, BG, EM) in the Chiltern population of *C. heterophylla,* using floral developmental stages 0 to 3 as the grouping variable and each pollination treatment as the test variables; the df = 3, N represents the number of measurements.

	Floral Stages	N	Mean Rank
Crossed (HC)	stage 0	30	46.60
	stage 1	30	69.60
	stage 2	30	78.22
	stage 3	30	47.58
	Total	120	
Bagged (BG)	stage 0	30	101.17
	stage 1	30	54.35
	stage 2	30	49.23
	stage 3	30	37.25
	Total	120	
Selfed (HS)	stage 0	30	50.65
	stage 1	30	56.22
	stage 2	30	85.27
	stage 3	30	49.87
	Total	120	
Emasculated (EM)	stage 0	30	82.88
	stage 1	30	83.55
	stage 2	30	39.28
	stage 3	30	36.28
	Total	120	

Table 4.17 Kruskal-Wallis H test showing the test statistics of pollination treatments (HC, HS, BG, EM) in the Chiltern population of *C. heterophylla,* using floral developmental stages 0 to 3 as the grouping variable and each pollination treatment as the test variables; the df = 3.

	Crossed (HC)	Bagged (BG)	Selfed (HS)	Emasculated (EM)
Chi-Square	19.003	59.052	21.095	51.870
df	3	3	3	3
P	<0.001	<0.001	<0.001	<0.001

The Kruskal-Wallis H test statistics computed illustrates that the variation in mean ranks observed for all floral developmental stages under the four pollination treatments are significantly different at df = 3 (X^2 – HC = 97.585, P < 0.001; X^2 – BG = 92.704, P < 0.001; X^2 – HS = 88.124, P < 0.001; X^2 – EM = 66.717, P < 0.001) [Table 4.19]. Therefore, flower longevity in both the Chiltern and the Norway populations studied is significantly different across floral developmental stages under different pollination treatments.

In order to identify where the differences in floral longevity among the four pollination treatments and across the four developmental stages lay in the Chiltern population, the *Post hoc* Dunnett T3 test is used. The result shows that flower longevity across all floral developmental stages do not show any significant difference in the crossed pollination treatment except between stages 0 and 1 (mean difference = 0.757, P = 0.044). Stages 0 and 2 differ significantly with mean difference = 0.969, P = 0.002, and stages 2 and 3 differ significantly with mean difference = 0.900, P = 0.005. However, in contrast, flower longevity is significantly different across all floral developmental stages in the bagged pollination treatment except between stages 1 and 2 (mean difference = 0.304, P = 0.810) and stages 2 and 3 (mean difference = 0.328, P = 0.774). But the difference between stages 1 and 3 is marginal, with mean difference = 0.633, P = 0.046. In the selfed pollination treatment, floral longevity is not significantly different across the floral developmental stages except between stages 0 and 2 (mean difference = 1.591, P < 0.001), stages 1 and 2 (mean difference = 1.346, P = 0.001) and stages 2 and 3 (mean difference = 1.512, P < 0.001). The result obtained shows that the floral longevity in the emasculated pollination treatment is significantly different across all floral developmental stages except between stages 0 and 1 (mean difference = 0.021, P = 1.00) and stages 2 and 3 (mean difference = 0.078, P = 1.00) [Appendix 2.1].

Table 4.18 Kruskal-Wallis H test showing the mean ranks of pollination treatments (HC, HS, BG, EM) in the Norway population of *C. heterophylla,* using floral developmental stages 0 to 3 as the grouping variable and each pollination treatment as the test variables; the df = 3, N represents the number of measurements.

	Floral Stages	N	Mean Rank
Crossed (HC)	stage0	30	95.27
	stage1	30	85.70
	stage2	30	41.28
	stage3	30	19.75
	Total	120	
Bagged (BG)	stage0	30	105.50
	stage1	30	69.35
	stage2	30	42.17
	stage3	30	24.98
	Total	120	
Selfed (HS)	stage0	30	88.78
	stage1	30	91.07
	stage2	30	35.32
	stage3	30	26.83
	Total	120	
Emasculated (EM)	stage0	30	81.60
	stage1	30	86.55
	stage2	30	46.15
	stage3	30	27.70
	Total	120	

Table 4.19 Kruskal-Wallis H test showing the Test Statistics of pollination treatments (HC, HS, BG, EM) in the Norway population of *C. heterophylla,* using floral developmental stages 0 to 3 as the grouping variable and each pollination treatment as the test variables; The df = 3.

	Crossed (HC)	Bagged (BG)	Selfed (HS)	Emasculated (EM)
Chi-Square	97.585	92.704	88.124	60.717
df	3	3	3	3
P	<0.001	<0.001	<0.001	<0.001

To further examine where the difference in floral longevity among the four pollination treatments and across the four developmental stages lay in the Norway population, the *Post hoc* Dunnett T3 test is employed.

The result obtained shows that, in the crossed pollination treatment, stages 0 and 1 are significantly different from stages 2 and 3; with mean difference between stage 0 and 2 = 1.744, P < 0.001; stages 0 and 3 = 2.277, P < 0.001; stages 1 and 2 = 1.543, P < 0.001; stages 1 and 3 = 2.076, P < 0.001. Also stages 2 and 3 are significantly different with mean difference = 0.533, P < 0.001. in addition, floral longevity is significantly different (P < 0.001) across all floral developmental stages in the bagged pollination treatment. Similar results are obtained in the Selfed and emasculated pollination treatments where, significant differences are recorded among all floral developmental stages except between stages 0 and 1(HS - mean difference = 0.089, P = 0.991; EM – mean difference = 0.145, P = 0.887); and stages 2 and 3 (HS - mean difference = 0.187, P = 0.281; EM – mean difference = 0.444, P = 0.039) [Appendix 2.2]. It is observed that the difference between stages 2 and 3 in the emasculated treatment is only marginal.

4.3.6 Effect of Autonomous Selfing on Flower Longevity in Chiltern and Norway Populations

To compare the variations observed in the autonomous (bagged – BG) pollination treatments between the populations of *C. heterophylla* studied (Chiltern and Norway), data are pooled from both populations and Mann-Whitney U test for two independent samples is applied. The results obtained demonstrate that bagged flowers (autonomous pollination) in Norway population senesced earlier than in the Chiltern population [BG – Chiltern = 175.89; BG – Norway = 65.11] (Table 4.20). The test statistics confirms that there is significant difference in the mean rank variation observed in Table 4.20 [Mann-Whitney test: U = 553.00, n_1 = 120, n_2 = 120, P < 0.001] (Table 4.21).

Table 4.20 Mann-Whitney U tests for two independent samples showing the mean ranks for Chiltern and Norway populations of *C. heterophylla* under bagged (BG) pollination treatment, using population as the grouping variable and the bagged pollination treatment as the test variable; the df = 1, N represents the number of measurements.

	Population	N	Mean Rank	Sum of Ranks
Bagged (BG)	Chiltern	120	175.89	21107.00
	Norway	120	65.11	7813.00
	Total	240		

Table 4.21 Mann-Whitney U tests for two independent samples showing the Test Statistics for Chiltern and Norway populations of *C. heterophylla* under bagged (BG) pollination treatment, using population as the grouping variable and the bagged pollination treatment as the test variable; the df = 1, N represents the number of measurements.

	Bagged (BG)
Mann-Whitney U	553.000
Wilcoxon W	7813.000
Z	-12.382
P	<0.001

Examining the bagged (BG) pollination treatment at each floral developmental stage reveals that the flowers at stage 0 of development live longer than at stages 1, 2, and 3. The variations in mean ranks observed at stage 0 = 172.05, stage 1 = 120.82, stage 2 = 102.35 and stage 3 = 86.78 (Table 4.22). Kruskal-Wallis test statistics shows that this variation in mean ranks is significantly different among floral developmental stages (Kruskal-Wallis U = 51.518, df = 3, P < 0.001) [Table 4.23].

Table 4.22 Kruskal-Wallis H test for several independent samples showing the mean ranks for floral developmental stages of Chiltern and Norway populations of *C. heterophylla* under bagged (BG) pollination treatment, using floral developmental stages as the grouping variable and the bagged pollination treatment as the test variable. The df = 3, N represents the number of measurements.

	Floral Stages	N	Mean Rank
Bagged (BG)	stage 0	60	172.05
	stage 1	60	120.82
	stage 2	60	102.35
	stage 3	60	86.78
	Total	240	

Table 4.23 Kruskal-Wallis H test for several independent samples, showing the test statistics for floral developmental stages in Chiltern and Norway populations of *C. heterophylla* under bagged (BG) pollination treatment; using floral developmental stages as the grouping variable and the bagged pollination treatment as the test variable. The df = 3, N represents the number of measurements.

	Bagged (BG)
Chi-Square (X^2)	51.518
df	3
P	<0.001

Overall, the Norway population showed shorter flower longevity of 4.22±1.47days for bagged (BG) pollination treatment than the Chiltern population with 7.76±1.15days (Table 4.2 and Table 4.3). The results of the two populations analysed above have revealed that the Norway population can be described as an early selfers (prior selfing), while the Chiltern population can be described as a late selfers (delayed selfing). This suggests that variation in autonomous selfing is related to the size of flowers between *C. heterophylla* populations and has implications for reproductive success under unpredictable pollinator visitation.

4.4 Discussion

This chapter has focused on the effect of pollination treatments on floral longevity in the two study populations of *Collinsia heterophylla*. Although there has been a significant theoretical and empirical interest in the evolution of flower longevity as it relates to mating system evolution in plants, relatively little work has focused on the relationships between flower longevity and mating system (Karle and Boyle, 1999; Sato, 2002; Weber and Goodwillie, 2007). In *C. heterophylla,* studies of mating systems have been mainly directed on floral traits associated with mating systems as well as the cost and benefit of male and female fitness. This study provides insights into the effect of pollination and flower longevity on the mating system of *C. heterophylla*.

The two populations studied (Chiltern and Norway) were found to respond differently to each of the four pollination treatments under the same greenhouse conditions. This corroborates the result obtained by Rogers, (2006) who showed that flowers have a limited life span and that floral longevity is species-specific and largely independent of environmental factors. Therefore, the results from this study provide a positive answer to the question, "Does floral longevity differ among different populations of *C. heterophylla* studied under similar environmental conditions?" The results obtained show that flowers in the Chiltern population generally last longer than flowers in the Norway population (see Tables 4.2 and 4.3). The mean flower longevity in the Norway population ranged from 2.70±0.45sd to 9.24±0.82sd days, whereas in the Chiltern population, the flower longevity ranged from 4.84±1.09sd to 9.15±1.51 days across all floral developmental stages and pollination treatments.

In addition, the two populations showed significant difference in floral longevity under the four pollination treatments, as well as across the four developmental stages (see Tables 4.4 & 4.5 and Tables 4.6 & 4.7). It is therefore, evident from this study that the addition of pollen decreases flower longevity, while withholding of pollen increases flower longevity.

Flowers subjected to the emasculated pollination treatment were observed to live longer than the other treatments that received pollen. Although, the results obtained for the emasculated pollen treatment in both Chiltern and Norway populations of *C. heterophylla* studied were similar, it was observed that there were significant differences in the response of the two populations to emasculated pollination treatment across the four floral developmental stages (Table 4.8 & 4.9). The result for floral developmental stage was, however, expected as these stages occur over three to four days of anthesis.

In *C. heterophylla,* the ability to self pollinate vs. depend on pollinators (cross pollination) for successful reproduction has been associate with flower size and several other floral traits. Small flowers are associated with autonomous selfing while large flowers are associated cross pollination (Armbruster *et al.*, 2002). However, studies have shown that even in the outcrossing species, when pollinators are limiting, late selfing can ensure seed set (Weber and Goodwillie, 2007). The results obtained in this chapter agree with that obtained by Weber and Goodwillie (2007), and also revealed that autonomous selfing limits flower longevity in both populations. The Norway population, which has small flowers, were observed to senesce earlier than the Chiltern population, which has large flowers. This result also corroborates the result obtained by Primack (1985), who observed that species of plants that undergo autonomous selfing commonly have shorter-lived flowers than plant species of the same genus, species or family that undergo outcrossing. He then suggested that selfing species may profit from pollinating faster and moving on to develop fruits, while an outcrossing species allows flowers to remain open for a longer time and hence the likelihood of pollinator visitation, resulting in increased plant vigour (Weber and Goodwillie, 2007).

Furthermore, the results obtained from this study showed that in both populations flowers senesced earlier with cross pollination than self pollination. The difference between these treatments was found to be significant (see Tables 4.12 & 4.13 and Tables 4.14 & 4.15). This suggests

that pollen grains germinated and in turn grew faster on the cross styles than on self styles, showing a form of partial cryptic incompatibility in this species (see Eberhard and Cordero, 1995). In addition, the results obtained show that, without manipulation and with the elimination of pollinators, the timing of autonomous selfing (bagged (BG) pollination treatment) significantly affects the rate of flower senescence in both populations of *Collinsia heterophylla.* For example, autonomous selfing triggered earlier flower senescence in the Norway population (mean days = 4.22±1.47s.d) than in the population obtained from Chiltern in UK (mean days = 7.76±1.15s.d). This agrees with the result obtained by Weber and Goodwillie (2007), who showed that autonomous selfing reduces flower longevity in *Leptosiphon jepsonii,* and is also consistent with experiments reported on other plant species (see Underwood *et al.*, 2005; Rogers, 2006).

The flowers of the Chiltern population generally lasted longer than the flowers of the Norway population. Flower longevity in both populations was shortened by pollination, whether cross, self, or open pollinated. This is consistent with the findings of Wyatt (1984), Primack (1985), Ritland and Ritland (1989) and Dole (1992), who reported that, for outcrossing species, increased intensity of pollination shortens flower life and, consequently, reduces the cost of floral maintenance. Therefore in late-selfing flowers, whenever cross-pollination is achieved, floral longevity and selfing rate is decreased by terminating flowering before autonomous self-pollination occurs. Thus whenever pollination intensity is high, autonomous self-fertilisation does not occur (Sato, 2002) [see Tables 4.2 and 4.3]. Hence, the ability or timing of autonomous self-fertilisation should largely influence both the selfing rate and floral longevity, because fewer ovules should remain unfertilized after an extended period of outcrossing.

This study was embarked upon because flowers are the main tool for mating system studies, and flower longevity has been found to be a limiting factor in plant mating system studies. Therefore, the ability of flowers to live long to be pollinated is a factor that has great consequences

for the evolution of species. However, in *C. heterophylla*, no previous study has documented the effect of pollination/fertilisation on flower longevity for mating system studies. Although, the two populations studied differ in their ability for autonomous selfing, this result is not conclusive as the population size used is relatively small. Also the populations used are not completely inbred lines; therefore do not provide a direct comparison of phenotypes. Future experiments may be improved by establishing experimental gardens of known phenotypes in the field to examine rates of senescence in natural pollinator environments. Also, a complementary approach has been suggested for assessing the source of pollen discounting. This involves genetic marker studies that compare outcross paternity of early and late selfing phenotypes in experimental arrays (Kohn and Barrett, 1994; Chang and Rausher, 1998; Fishman, 2000). This can be carried out using the restriction fragment length polymorphism (RFLP) technique.

4.5 Conclusion

In this study of the effects of pollination on flower longevity, I found that the pollination treatment, the time of pollen arrival (i.e. floral developmental stage), and population source all had significant effects on the number of days from anthesis to flower senescence. This study revealed that, although all *Collinsia* species are self compatible and exhibit a mixed mating system, populations studied could be distinctly grouped into generally selfing (Norway) and mostly outcrossing (Chiltern). However, the Chiltern (large-flowered) population studied was able to undergo autonomous selfing in the absence of pollinators; but this autonomous selfing occurred later than that observed in the Norway (small-flowered) population. It has been suggested that autonomous self pollination provides reproductive assurance in self-compatible hermaphrodite species, after a flower has been receptive to outcross pollen for several days. Therefore, in order to investigate if autonomous selfing achieves reproductive assurance as models suggest a further study will need to be carried out to examine the quantity and quality of seeds produced. In

addition to the experiment in chapter 4, a further experiment was carried out in chapter 5 to assess pollen performance in the two populations studied. This is because the crossed (HC) pollination treatment in the Norway population was observed to have the shortest flower longevity (1.62±0.30s.d), suggesting that crossed-pollen (i.e. pollen from Chiltern flower) fertilised ovules in Norway flower faster. Figueroa-Castro (2009) has reported that the abilities of pollen grains to germinate and develop pollen-tubes to convey the male gametes to fertilise the ovules vary between the self and outcross donors, and this can consequently can influence the evolution and breeding systems of the angiosperms. Moreover, several studies have reported that outcross pollen-tubes have an advantage over self pollen-tubes when growing in the style (see Bateman, 1956; Bowman, 1987; Casper *et al.*, 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al.*, 1990; Cruzan and Barrett, 1993; Rigney *et al.*, 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006; reviewed in Figueroa-Castro and Holtsford, 2009). Therefore, in the final chapter of this thesis (chapter 5), pollen germination and pollen-tube growth (*in-vitro and in-vivo*) are investigated to compare pollen-tube growth rate between the two study populations of *C. heterophylla*.

CHAPTER 5

5.0 Post-Pollination Mechanism in *Collinsia heterophylla*: Pollen-Tube Growth Rate

5.1 Introduction

In plants, the success of the pollen donor depends on several factors; one major factor is the post-pollination mechanism (Snow and Spira, 1991, 1996; Montalvo, 1992; Walsh and Charlesworth, 1992; Rigney *et al.*, 1993; Carney *et al.*, 1994; Eckert and Allen, 1997; Kruszewski and Galloway, 2006; reviewed in Figueroa-Castro, 2009). Post-pollination mechanisms, such as pollen-tube germination is a significant feature in angiosperms, which determines the genotype of the progeny (Figueroa-Castro, 2009). Although, a mixture of self and outcross pollen can be delivered to the stigma of a flower, the abilities of these pollen grains to germinate and develop pollen-tubes to convey the male gametes to fertilise the ovules in the ovary vary. These processes determine the siring success for self and outcross donors and consequently, can influence the evolution and breeding systems of the angiosperms (Figueroa-Castro, 2009).

Post-pollination processes are particularly important in natural populations of inter-crossable sympatric species with overlapping flowering seasons and pollinator sharing. Post-pollination mechanisms can operate as isolating barriers between species and hence determine the evolutionary trajectory for each species (Carney *et al.*, 1994). Therefore, by determining both pollen-tube growth rate and offspring paternity, it may be possible to separate post-pollination from post-zygotic diversity (Bateman, 1956; Walsh and Charlesworth, 1992; Eckert and Allen, 1997; reviewed in Figueroa-Castro and Holtsford, 2009).

Pollen-tube growth rate has been noted to be an essential characteristic of each plant species, in that it influences the competitive

abilities of pollen tubes when growing in the style (Snow and Spira, 1996). In addition, several studies have proposed that differences in pollen competitive ability among individual donors are large enough to affect siring ability (see Marshall and Folsom 1992; Snow and Spira 1996; Pasonen *et al.*, 1999; reviewed in Lankinen 2001). The siring success does not basically reflect the composition of pollen loads delivered on the stigmas, but mainly reveals the ability of each pollen grain to compete with other pollen grains delivered on the stigma at the same time. Therefore, the siring success and the paternity of the progeny reflect the abilities of pollen-tubes to travel down the style fast enough to deliver the male nuclei to the female gametophyte (see Montalvo, 1992; Rigney *et al.*, 1993; Carney *et al.*, 1994; Jones, 1994; Snow and Spira, 1996; Kruszewski and Galloway, 2006; reviewed in Figueroa-Castro, 2009).

In order to determine the advantage of outcross pollen over self pollen in terms of differential pollen-tube growth rate, it is necessary to demonstrate that differential siring success among pollen types is caused by differences in pollen-tube growth rate and not due to selective seed abortion (Bateman, 1956; Walsh and Charlesworth, 1992). Although, it was previously suggested that, when intra/inter-specific pollen mixtures are delivered on stigmas, it is likely that intra-specific pollen-tubes would be favoured for fertilisation to minimize hybridisation (Smith, 1968); this has been found to be only advantageous in the case where hybrids are less fit than the progeny from conspecific crosses (Carney *et al.*, 1994; Emms *et al.*, 1996).

Conversely, Jones (1994) proposed that if fertilisation is random, the siring success of self versus outcross donors can be predicted by the proportion of self versus outcross pollen deposited on the stigma. However, Pasonen *et al.* (2000) studied pollen-tube growth rate and seed-siring success among *Betula pendula* clones and their results showed that fertilisation is not random and that pollen competition takes place. They observed that the seed-siring success of two competing pollen donors was unequal in 20 of 29 cases and there was a significant positive correlation between seed-siring success and pollen-tube growth rate *in-vivo* and *in-*

vitro. Similarly, Colling *et al.* (2004) have reported that the ability of the plant *Scorzonera humilis* to produce seeds with higher vigour depended both on the density (pollen load) and source of pollen on the stigma. They observed that seed set increased with local conspecifics, but higher cross pollen loads increased the survival of the offspring; thereby suggesting that higher pollen loads increased pollen competition and the selectivity among gametes. Therefore, it appears that siring success does not only depend on the source of the pollen but also on the amount of pollen deposited on the stigmas. Colling *et al.*, (2004) further observed that adding pollen from a different population strongly increased progeny fitness compared with both natural pollination and pollen supplement from the same population.

Several studies have reported that outcross pollen-tubes have an advantage over self pollen-tubes when growing in the style (see Bateman, 1956; Bowman, 1987; Casper *et al.*, 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al.*, 1990; Cruzan and Barrett, 1993; Rigney *et al.*, 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006; reviewed in Figueroa-Castro and Holtsford, 2009). Consequently, outcross pollen-tubes are expected to fertilise more ovules and have a higher reproductive success than self pollen-tubes, especially in self-incompatible species (Bateman, 1956; Mulcahy, 1979; Lassere *et al.*, 1996; Figueroa-Castro and Holtsford, 2009).

Moreover, pollen-tube growth rate has been reported to be correlated with pollen size and/or length of style (see Aizen *et al.,* 1990; Williams and Rouse, 1990; Diaz and Macnair, 1999; Lee *et al.*, 2008, reviewed in Figueroa-Castro, 2009). Also, Lee *et al.* (2008) have suggested that, if pollen-tube growth is correlated with style length, and the sympatric species have contrasting style lengths, then pollen tubes from the long-style species are supposed to have an advantage in fertilising ovules of a short-style species; consequently, asymmetric hybridisation will be common (Diaz and Macnair, 1999; and see also Lee *et al.*, 2008; and Figueroa-Castro and Holtsford, 2009).

Furthermore, it has been suggested that pollen performance is not only affected by various incompatibility effects of the recipient plant, but also by environmental conditions. For example, the effects of temperature on pollen traits have been appropriately documented in several species. In addition, temperature during pollen development has been observed to influence the chemical composition of pollen, pollen viability and pollen-tube growth rate (see Pfahler, 1967; Sarr *et al.*, 1983; Fenster and Sork, 1988; Charlesworth *et al.*, 1990; Cruzan, 1990; Johnston 1993; Hormaza and Herrero, 1996, reviewed in Lankinen, 2001). However, Lankinen (2001) argued that, although a large number of studies have investigated the influence of environmental factors on pollen performance, the focus has rarely been on the consistency of individual pollen donors across environmental conditions, that is, testing for genotype-by-environment interactions (see Travers 1999; Lankinen 2000; Pasonen *et al.*, 2000). She then suggested that such interactions may demonstrate a potential for different plastic responses in the pollen-tube growth rate.

The great inter-specific and intra-specific diversity of floral morphology in *Collinsia heterophylla,* including style length variation in small versus large flowers, has made this group of plants an ideal model system for studying the significance of variation in pollen-tube growth rate and the evolution of mating systems. *Collinsia heterophylla* is self-compatible, but the timing of self-fertilisation can vary greatly among populations depending on the floral size and morphology (Armbruster *et al.*, 2002). However, one major question to be addressed is: when a mixture of self and cross pollen is loaded on a stigma, which pollen will have advantage over the other, self- or cross-pollen, and pollen from large- or small-flowered sub-populations? Lankinen and Armbruster (2007) have observed that, in *Collinsia heterophylla,* inbreeding depression was lower when large pollen loads were applied (11%) relative to the low pollen-load treatment (28%). In addition, the reduction was significant for two fitness components relatively late in the life-cycle: number of surviving seedlings and pollen-tube-growth rate *in-vitro*. The knowledge of the pollen-tube growth rate will enable ecological geneticists to predict the mating-system

evolution in *C. heterophylla* populations growing in the wild. Although, inter-specific pollinations might determine the degree of hybridisation and gene exchange among taxa, few studies have been carried out to investigate their frequencies and consequences (see Diaz and Macnair, 1999; Chapman, *et al.*, 2003; Figueroa-Castro and Holtsford, 2009; Montgomery, *et al.*, 2010). Also, there is a lack of literature that investigates the importance of post-pollination mechanisms and their consequences for mating system evolution in *C. heterophylla.* However, Lankinen, *et al.* (2009), have reported the results of the pollen-tube growth rate in the large- and small-flowered *C. heterophylla* that were selected from the same population (that is, Southern California (Norway) population). They reported that there was no significant difference in the pollen-tube growth rate of the large-flowered compared to the small-flowered plants within this population. But they did not compare across different populations.

Therefore, the experiment in this chapter investigates the post-pollination mechanisms (that is, the pollen-tube growth rates) in *C. heterophylla* populations obtained from two different geographical regions. The two populations of *C. heterophylla* used in this study are the Northern California population via Chiltern seeds, UK, and the Southern California population via Norway. The aim of this study is to examine the pollen-tube growth rate *in-vivo* and *in-vitro* in these two populations of *Collinsia heterophylla:* population 1 – via Norway (mostly small-flowered) and population 2 – via Chiltern (mainly large-flowered). The objectives of this study are:

a) To investigate the differences in pollen-tube growth rate between Norway and Chiltern populations (small and large flowers respectively) growing on a growth medium.
b) To assess the differences in pollen tube-growth rate for self pollen growing in the styles (small or large flowers).
c) To examine the pollen-tube growth rates for pollen growing in the styles of different flowers from other populations (that is pollen from

small flowers growing on the large-flowered style and pollen from the large flowers growing on the small-flowered style).

d) To investigate and discuss the likelihood of pollen competition and hence differential siring success of outcross versus self pollen, and the consequences of inter-population hybridisation when two sources of pollen (outcross- versus self-) interact in the styles of each of the two populations studied.

The questions to be answered in this chapter include:

i. Does *in-vitro* and *in-vivo* pollen-tube growth rate differ within and between populations of *C. heterophylla?*

ii. Is there any difference in pollen-tube growth on self- versus cross-styles for the two populations of *C. heterophylla* studied?

iii. What are the implications of different pollen-tube growth rates for mating systems evolution?

5.2 Materials and Methods

5.2.1 Study Species

The study species, *Collinsia heterophylla* Buist (Plantaginaceae), is a diploid, self-compatible annual. However, the timing of self-fertilisation varies greatly among populations depending on the floral size and morphology (see Kalisz *et al.*, 1999; Armbruster *et al.*, 2002). At four days after flower opening, the style length for the Norway population (including the stigma) is 13.2± 0.96 mm (± s.d; n = 19; Lankinen *et al.*, 2009). Pollen is binucleate (Schrock and Palser, 1967) and ovaries develop into dry, dehiscent seed capsules (Lankinen *et al.*, 2009).

The stigma of *Collinsia heterophylla* is very small and according to Lankinen *et al.* (2009), a rough estimation shows that at least 50 pollen grains can be in direct contact with the stigmatic surface at the same time and the stigma can hold more pollen in additional layers. As a result there may often be about five times as much pollen on the stigma as ovules to fertilise (that is, approximately 5 pollen: 1 ovule), and thus pollen competition can probably occur. This is because at any point in time when the stigma has a full pollen load, there is more pollen on the stigma than

the amount of ovules to be pollinated. Thus, *C. heterophylla* is an ideal system for this study.

The parental families used in this study were obtained from two different locations. One population had pale-purple flowers and were obtained from the Norwegian University of Science & Technology, Trondheim (NTNU); but originated from a population in Sisar Canyon, Ventura County; Southern California. This population is referred to as the Norway population and is generally small-flowered. The second population had deep-purple flowers and was obtained from Chiltern seeds, Bortree Stile, Ulverston, Cumbria, England. They are believed to have come from Northern California. This second population is referred to as the Chiltern population and is mostly large-flowered. Seeds from the two populations were further selfed over two generations in the greenhouse (2006 to 2008) to produce two near to pure inbred lines of Norway and Chiltern populations. The two populations were chosen because there seems to be a clear difference in flower size between them

5.2.2 Pollen-Tube Growth Rate

5.2.2.1 Measurement of Pollen-Tube Growth Rate in Germination Medium

In order to reduce the effect of the female tissue on pollen germination, the *in-vitro* pollen tube growth was estimated. However, the result of *in-vitro* experiments may not always be representative of natural processes, because the growth medium environment is not the same as the stigma and style environment (Lankinen *et al.*, 2009).

To investigate if pollen grains in small-flowered and large-flowered populations differ in pollen-tube growth rate, and to compare the pollen-tube growth rate in the growth medium (*in-vitro*) with the pollen-tube growth rate on the stigma, pollen grains were germinated in Hoekstra medium (Hoekstra and Bruinsma, 1975). The Hoekstra medium was prepared following the method described by Lankinen *et al.* (2009; see Appendix 3.0 for recipe). Pollen grains were collected from 20 small-flowered (Norway population) and 20 large-flowered (Chiltern population)

plants. Pollen grains from two freshly dehisced anthers per plant were sprinkled sparsely onto a drop of medium on a glass slide and were left in a dark chamber at a constant temperature of 20 - 21°C for two hours to allow the pollen-tubes to germinate. Prior to measurements, pollen-tube growth was arrested using concentrated glycerol for 2 hrs from the time of treatment (pollen dusting), as it has been previously observed that when pollen-tubes were left to grow overnight, they continued to grow and became longer than the length of the pistil (reviewed in Lankinen *et al.*, 2009; Lankinen and Armbruster, pers. com.). Therefore, pollen-tube growth was arrested in order to reduce errors due to continued pollen-tube growth during measurements. Also, so that the effect of germination time on the metric will be reduced, pollen-tubes shorter than 0.12mm were not included in the measurement as they were assumed to have germinated later than others (see Lankinen *et al.*, 2009).

To estimate *in-vitro* pollen-tube growth rates, the lengths of ten pollen tubes per sample were measured under a light microscope. The unit of measurement used was micrometers (microns); this was then converted to millimetre. Because pollen tubes were visible under the light microscope, there was no need for staining at this stage (Fig. 5.1A). The means of the ten pollen tubes measured per plant for 20 Norway plants and 20 Chiltern plants were calculated.

5.2.2.2 Measurements of Pollen-Tube Growth Rate in the Style

To investigate the pollen development on the pistil of small as well as large flowers, pollen-tube germination was assessed in receptive pistils (2 - 3 days after flower opening) across 40 maternal plants (20 small-flowered Norway and 20 large-flowered Chiltern). This is because the result for stigma receptivity corroborates the findings of Armbruster *et al.*, (2002) who has reported that stigma receptivity in *Collinsia heterophylla* is higher at 3 days after flower opens corresponding to when the third anther has dehisced. The growth rate of pollen tubes was determined for each population through a single donor hand-pollinations conducted in the greenhouse.

First, hand pollinations were carried out within each population (selfing) and second, hand pollinations were carried out between the two populations (inter-population crosses). In the selfing pollination experiments within each population, each plant served as a pollen donor as well as a recipient of its own pollen. Pollen grains were placed randomly on stigmas of flowers at stage 2, but care was taken to ensure that only pollen grains collected from flowers of the same plant were used to self the recipient flower on the same plant. This procedure was meticulously carried out for each population.

Table 5.1 Experimental design with twenty randomly selected plants from each of the two populations (Norway – n = 20 and Chiltern – n = 20).

Dam **Sire**	**Norway/small-flowered**	**Chiltern/large-flowered**
Norway/small-flowered	20 (selfed)	1 Norway X 20 Chiltern (inter-population cross)
Chiltern/large-flowered	1 Chiltern X 20 Norway (inter-population cross)	20 (selfed)

In the selfing experiment, three to four flowers per plant were emasculated at stage 3 (i.e. third/fourth day of anthesis), these were labelled as the recipient flowers. These flowers were hand pollinated with pollen grains from other flowers on the same plant, that is 4 flowers X self pollen – collected from flowers (donors) on the same plant as the recipient within the same population (Norway X Norway and Chiltern X Chiltern). In the cross pollination experiment, four flowers on each of 20 recipient plants in each population were emasculated at stage 2 (i.e. third day of anthesis); then, a single donor plant was selected from each population to act as the pollen donor for all the twenty plants within the same population (see design in Table 5.1). Thus, four flowers (4 recipients) were cross pollinated with pollen from the other population. Pollen donors were randomly chosen, and only one pollen donor was used to pollinate the recipient plant. This was done in order to eliminate the differences that

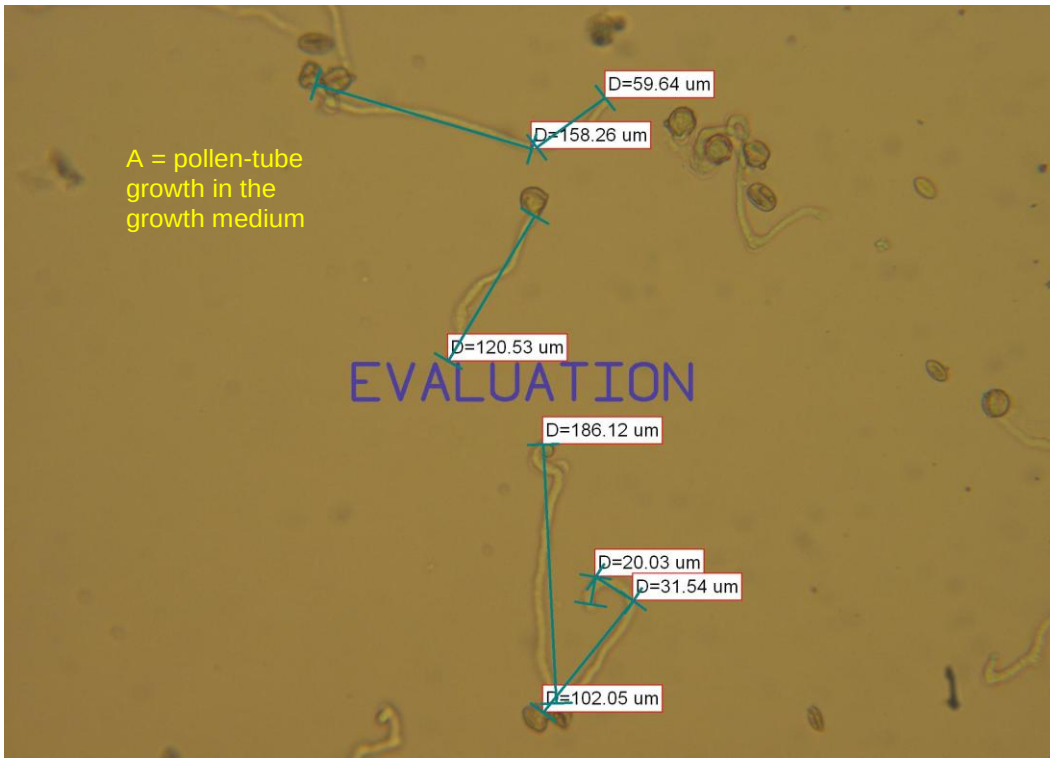
could result if pollen grains from different sources were used to pollinate a single (recipient) flower. This was repeated for all the 20 plant in each population.

All flowers were hand pollinated on the same day to reduce environmental variance, e.g. temperature and humidity. The stigma of each emasculated flower was pollinated with pollen grains from 2-3 recently dehisced anthers. The time of pollination was recorded as it is important to allow equal time for pollen-tube growth, so as to reduce variance caused by different growth periods. The experiment was conducted in the greenhouse of the University of Portsmouth, UK, in May 2009.

Styles were collected two hours after pollination. According to Lankinen *et al.*, (2009) *C. heterophylla* pollen grains germinate on the stigma 1 - 1.5 (2) hrs after pollination. Pollinated styles of selfed and crossed flowers were collected into labelled small 'Bijous' disposable bottles containing 70% ethanol for fixation. Fixed flowers were left in their containers (bijous) and stored in the cold room overnight (approximately 16 hours).

5.2.3 Staining Technique

To stain and view pollen tubes for measurement, fixed styles were removed from the fixative (70% ethanol) and thoroughly rinsed with distilled water, tissues were softened in 1 M NaOH for 2–3 hrs, and then thoroughly rinsed under distilled water (two to three times). Styles were then placed on glass slides (labelled), and stained for at least 3 hours in two to three drops of 0.1% (w/v) aniline blue dye solution in aqueous K_3PO_4, pH 6.8 and then squashed (Kho and Baer, 1968; reviewed in Figueroa-Castro, 2008; see Appendix 3.1 for method of preparation). The presence of pollen-tube in the style was evident under the UV epifluorescence light mounted on a Leitz Laborlux - S light microscope in the School of Biology Sciences, University of Portsmouth, UK.



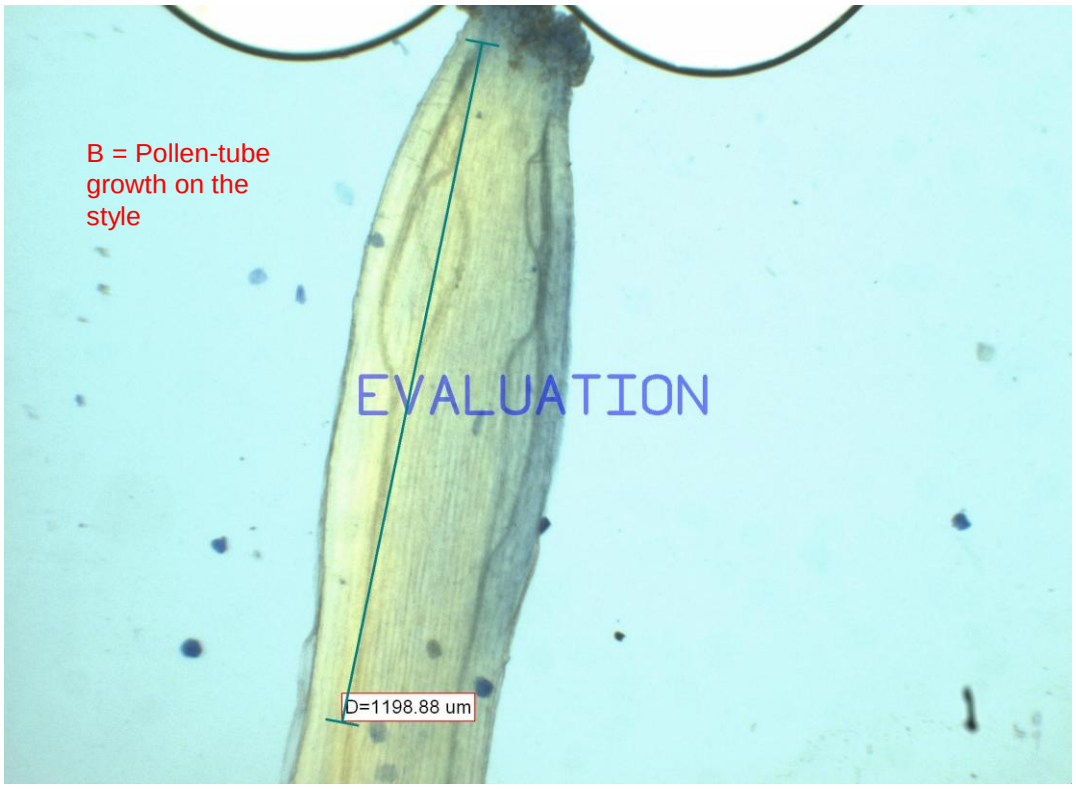


Figure 5.1A & B Pollen-tube growth (A) in the growth medium – Hoekstra medium [x10 objective] and (B) in the style of *Collinsia heterophylla* [x20 objective].

The length of the longest pollen-tube was measured under UV light; pollen-tube was measured from the centre of the stigma to the tip of the longest pollen tube (Fig. 5.1B). It was not possible to take photographs under the UV light as it was too dim. The maximum rather than the mean pollen-tube length per sample was used for the analysis in this experiment. This is because the growth of pollen-tubes on the style is influenced by several factors (e.g., endogenous as well as exogenous pollen environment, competitive ability etc), and only the first pollen-tube to reach the ovule fertilises the ovule. Therefore, mean pollen-tube growth will not reflect the optimum pollen-tube growth ability.

Pollen-tubes were measured with the aid of mounted eye piece micrometer. The micrometer was calibrated to obtain millimetre readings for the pollen-tube lengths. The *in-vivo* pollen-tube value that was used for this investigation was the mean value of the longest pollen-tube (converted to millimetres) measured in the pistils of 4 selfed and 4 crossed for 20 Chiltern and 20 Norwegian plants. Pollen-tube growth treatments were applied to 160 flowers per population (4x20 self and 4x20 cross per population). Consequently, the mean measurement for pollen-tube of four flowers with the same treatment on the same plant was used for the purpose of the statistical analysis.

5.2.4 Statistical Analyses

Data collected were summarised into mean, standard deviation and standard error of mean using descriptive statistics. Prior to the application of statistical tests to the data, normality and homogeneity tests were computed, as in chapter three using SPSS 16.0 (SPSS, 2008). The results of the normality and homogeneity tests indicated that data from both *in-vitro and in-vivo* experiments did not meet parametric assumptions (Appendix 3.2). Therefore, non-parametric tests were used to analyse the data. All analyses were carried out using SPSS 16.0 (SPSS, 2008).

5.2.4.1 Pollen-Tube Growth in the Hoekstra Medium

To test for differences in pollen-tube growth rate in the growth medium for the Norway and Chiltern populations, the Mann-Whitney U test statistics for two independent samples was computed.

5.2.4.2 Pollen-Donor Behaviour on the Recipient Style (Selfing vs. Inter-Population Cross)

To assess the difference in pollen-tube growth rates of pollen-donors in the style of recipient flowers of Chiltern and Norway population, a graphical comparison (error bar plot) was used to show the distribution in mean pollen-tube lengths. The error bar plot showed that there are differences in the selfed and crossed pollination treatments in the two populations (Chiltern and Norway) studied. To test if the variation observed was significantly different among the groups, a Kruskal-Wallis H test for several unrelated samples was computed. Because the test showed that the pollination treatments were significantly different, a DunnettT3 *post hoc* test was used to find out where the difference lay.

5.2.4.3 Pollen-Tube Growth Rate *In-vivo* and *In-vitro*

To investigate the relationship between the pollen-tube growth rate in the growth medium and in the style (*in-vitro* and *in-vivo*), data were pooled from related pistils but different pollination treatments (i.e. Norway selfed and crossed as well as Chiltern selfed and crossed). This was done in order to obtain a comparatively large enough sample in the *in-vivo* germination treatment as well as in the *in-vitro* pollen-tube growth for each of the two populations studied. The descriptive statistics showed variation in means and standard deviation between the Norway and Chiltern populations. This variation was assessed using the Mann-Whitney U test for two independent samples; the germination types (1 = *in-vitro,* 2 = *in-vivo*) as the grouping variable and the mean pollen-tube (Norway and Chiltern) lengths as the test variables. There was significant difference, but this could not be tested further using a DunnettT3 *post hoc* test because the grouping variable is less than three.

5.4 Results

5.4.1 Differences in Pollen-tube Growth Rate in (a) Hoekstra Germination Medium and (b) Style

Generally, when slides were observed under the microscope, it was found that about 80 – 90% of the pollen on the medium had germinated two hours (2hrs) after pollen were deposited on the medium. Although pollen from both populations showed very high germination rate in the Hoekstra medium, the mean pollen-tube growth rate of the two populations per time was slightly different. The mean pollen-tube length in the Chiltern population was slightly greater than in the Norway population (Table 5.2).

Table 5.2 Descriptive statistics showing the mean, std. deviation, std. Error of the mean, minimum and maximum pollen-tube length for the two populations of *C. heterophylla* studied (Norway and Chiltern) in the growth medium; N = 50.

Population	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Chiltern	0.295	0.099	0.014	0.152	0.552
Norway	0.259	0.059	0.008	0.123	0.425

To examine if this difference in mean was statistically significant, Mann-Whitney U test is used to test the variation. The result showed that there was no significant difference in the pollen-tube growth rate between study populations (Mann-Whitney U test = 1177.000, Z = 0.503, P = 0.615; N_1 = 50, N_2 = 50; Mean $Rank_{Norway}$ = 49.04, Mean $Rank_{Chiltern}$ = 51.96; Tables 5.3 & 5.4). However, the mean rank in the Chiltern population was slightly higher than in the Norway population.

Table 5.3 Mann-Whitney U test showing the mean ranks for the mean pollen-tube length in the two populations of *C. heterophylla* (Chiltern and Norway), using population as the grouping variable and mean pollen-tube length as the test variable; the degree of freedom = 1 and N represents the number of measurements.

	Population	N	Mean Rank	Sum of Ranks
Mean Pollen-Tube Length (mm)	Norway	50	49.04	2452.00
	Chiltern	50	51.96	2598.00
	Total	100		

Table 5.4 Mann-Whitney U test showing the test statistics (X^2) of the mean pollen-tube length (mm) for the two populations of *C. heterophylla* (Chiltern and Norway), using population as the grouping variable and mean pollen-tube length as the test variable; the degree of freedom = 1 and N represents the number of measurements.

	Mean Pollen-tube length (mm)
Mann-Whitney U	1177.000
Wilcoxon W	2452.000
Z	-0.503
Asymp. Sig. (2-tailed)	= 0.615

5.4.2 Differences in Pollen-Donor Performance in the Styles of Chiltern and Norway Populations

The results of the *in-vivo* germination treatment in the two populations show that there is significant difference in pollen-tube growth rate between the two populations under both selfing and inter-population crossing treatments (Tables 5.5; 5.6 & 5.7). The table of descriptive statistics (Table 5.5) shows the mean pollen-tube length for Chiltern and Norway population. The error bar plot shows no overlap whatsoever among pollination types. The Chiltern (large-flowered) pollen-tube growth was faster than the Norway (small-flowered) pollen-tube, both on the selfed as well as on the crossed styles (Fig. 5.2). In addition, it was

observed that the Chiltern flower pollen-tube grew fastest on the Norway flower than the Norway pollen, suggesting that when the two populations co-exist together in the same location and pollinators are in abundance, all flowers (Norway – small or Chiltern - large) are likely to be fertilised by the Chiltern (large-flowered) pollen.

Table 5.5 Descriptive statistics showing mean lengths (mm), standard deviation, and standard error of means for the two different pollination treatments *in-vivo* (Selfing and Crossing) in the two populations of *Collinsia heterophylla* studied (Norway and Chiltern).

Population and Crosses	*Mean*	*Std. Deviation*	*Std. Error*
Chiltern flower selfed	0.874	0.130	0.029
Chiltern flower x Norway pollen crossed	0.563	0.153	0.034
Norway flower selfed	0.411	0.701	0.016
Norway flower x Chiltern pollen crossed	1.196	0.236	0.053

Figure 5.2 shows that the pollen-tube growth rates differed between pollination treatments. Table 5.5 shows that the standard error of the mean was highest in the Norway flower crossed with the Chiltern pollen (Norway flower x Chiltern pollen crossed) but lowest in selfed Norway flower; while, the standard deviation was highest in selfed Norway flower. When the variations observed were tested with Kruskal-Wallis H test for several independent samples; the variations were found to be statistically significant at 0.05 CI level.

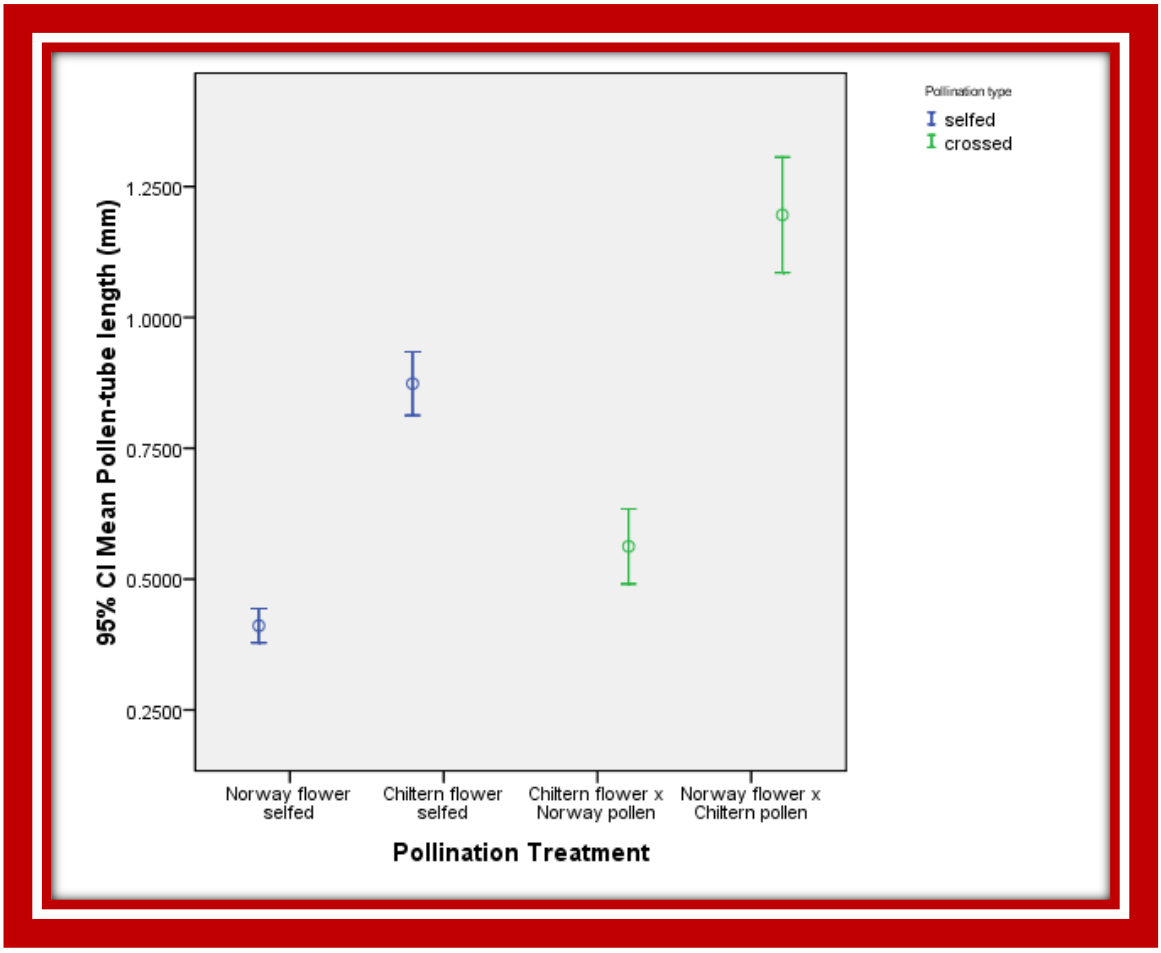


Fig. 5.2 Variations in mean pollen-tube growth length on selfed and on crossed styles of the two populations. Chiltern population = large-flowered and Norway population = small flowered; CI – 95%.

Table 5.6 Kruskal-Wallis H test showing the mean ranks of pollen-tube length for the two pollination treatments (selfed and inter-population cross) in the two populations of *C. heterophylla* (Chiltern and Norway); using pollination treatments as the grouping variable and mean pollen-tube length (mm) as the test variable. The df = 3, N represents the number of measurements.

	Pollination Treatment	**N**	**Mean Rank**
Mean Pollen-Tube Length (mm)	Norway flower selfed	20	14.30
	Chiltern flower selfed	20	51.72
	Chiltern flower x Norway pollen	20	28.25
	Norway flower x Chiltern pollen	20	67.72
	Total	80	

Table 5.7 Kruskal-Wallis H test showing the Test Statistics (X^2) mean pollen-tube length (mm) for the two pollination treatments (selfed and inter-population cross) the two populations of *C. heterophylla* (Chiltern and Norway); using pollination treatments as the grouping variable and mean pollen-tube length (mm) as the test variable. The df = 3, N = 40.

	Mean Pollen-Tube Length (mm)
Chi-Square	63.121
df	3
Asymp. Sig.	<0.001

Using a non-parametric *post hoc* test Dunnett T3 to determine the source of the difference, it was observed that three germination treatments were significantly different from each other at CI = 0.05, P < 0.001; but the difference between the Norway selfed and Chiltern flower x Norway pollen was significant at CI = 0.05, P = 0.003 (Appendix 3.3).

5.4.3 Differences in Pollen-Tube Growth Rate *In-vitro/ In-vivo* and Between-Populations

The results obtained show that the mean *in-vitro* and *in-vivo* pollen-tube growth rates differed greatly between the two populations of *Collinsia heterophylla* studied (Table 5.8). The descriptive statistics in Table 5.8 shows that the Chiltern population generally had higher pollen-tube growth rate *in-vivo* than the Norway population (Chiltern mean = 1.035±0.249s.d; Norway mean = 0.487±0.140s.d). However, it was observed that pollen-tube growth rate in the two populations were generally higher in the style (*in-vivo*) than in the growth medium (*in-vitro*) and this variation was found to be highly significant (Table 5.9).

Table 5.8 Descriptive statistics showing the mean, standard deviation, standard error, minimum and maximum values for the pollen-tube length from a pooled data of *in-vitro* and *in-vivo* germination treatments for the two populations of *C. heterophylla* studied (Norway and Chiltern); mean pollen-tube length is in millimetres (mm).

Population *Treatment*	Mean	Standard Deviation	Standard Error	Mini-mum	Maxi-mum
Chiltern					
in-vitro	0.295	0.099	0.014	0.152	0.552
in-vivo	1.035	0.249	0.039	0.640	1.624
Norway					
in-vitro	0.259	0.059	0.008	0.123	0.425
in-vivo	0.487	0.140	0.022	0.278	0.812

When the two germination types were compared within each population using Mann-Whitney U test for two independent samples, it was observed that the difference between *in-vitro* and *in-vivo* germination within each population was significantly different (Mann-Whitney U test Chiltern = 0.000, Z = -8.121, P < 0.001; Norway = 88.000, Z = -7.407, P < 0.001) [Table 5.10].

Table 5.9 Mann-Whitney U test showing the mean ranks of the pollen-tube length (mm) for the two germination treatments (in-vitro and in-vivo) in the two populations of *C. heterophylla* (Chiltern and Norway), using germination treatments as the grouping variable and pollen-tube length in each population the test variable; the df = 1, N represents the number of measurements.

Population	**Germination Treatment**	**N**	**Mean Rank**	**Sum of Ranks**
Chiltern Population Pollen-Tube Length (mm)	*in-vitro*	50	25.50	1275.00
	in-vivo	40	70.50	2820.00
	Total	90		
Norway Population Pollen-Tube Length (mm)	*in-vitro*	50	27.26	1363.00
	in-vivo	40	68.30	2732.00
	Total	90		

Table 5.10 Mann-Whitney U test showing the Test Statistics (X^2) of pollen-tube length (mm) for the two germination treatments (*in-vitro* and *in-vivo*) for the two populations of *C. heterophylla* (Chiltern and Norway), using germination treatments as the grouping variable and pollen-tube length as the test variable; the df = 1, N represents the number of measurements.

	Chiltern Population Pollen-Tube Length (mm)	Norway Population Pollen-Tube Length (mm)
Mann-Whitney U	0.000	88.000
Wilcoxon W	1275.000	1363.000
Z	-8.121	-7.407
Asymp. Sig. (2-tailed)	<0.001	<0.001

5.5 Discussion

Generally, the results of pollen-tube growth obtained from the germination treatments (*in-vitro* and *in-vivo*) varied enormously within and between the populations studied. However, the variation observed between populations in the growth medium experiment (*in-vitro*) was not statistically significant (Tables 5.2, 5.2 and 5.4). This suggests that using the results obtained for pollen-tube growth experiments in the growth media could be inaccurate and misleading. This is because; pollen grains have been reported to be very sensitive to their growth environment (see Lankinen, 2001). But according to Lankinen *et al* (2009), this method of assessing the pollen could be particularly important in a species with delayed stigma receptivity, where other options of assessing pollen performance excluding maternal influence are more difficult (see other references in Lankinen *et al*, 2009).

The results obtained from the *in-vivo* experiment revealed a very interesting pattern of pollen behaviour among the populations studied (Norway and Chiltern). It was observed that pollen from the same donor population (i.e. Norway or Chiltern) had very different pollen-tube growth on each of the selfed- and inter-population cross-pollination treatments carried out as shown in Tables 5.5, 5.6 and 5.7; Fig. 5.2. The variations in pollen-tube growth rate observed within and among populations are found

to be significantly different. There is therefore an indication that pollen-tube growth and germination could be highly influenced by the environment within which pollen is deposited. The results obtained in this experiment are consistent with the results of early researchers who reported that the environment in which the pollen germinates and grows (i.e. recipient plant) has the ability to greatly influence pollen-tube growth rate and the ability of the pollen to compete, hence leading to siring success (see Fenster and Sork, 1988; Cruzan, 1990; Johnston, 1993). According to Lankinen *et al.* (2009), this siring success could influence the evolution of plant species.

Also, in this study, it was observed that pollen from the Norway population generally showed shorter mean pollen-tube length than the pollen from the Chiltern population. The shortest mean pollen-tube length was recorded for the selfed Norway pollination treatment (i.e. Norway (small) flower x Norway pollen), followed by the Norway pollen crossed with Chiltern flower (i.e. Chiltern (large) flower x Norway pollen). Interestingly, the longest mean pollen-tube length was found when Chiltern pollen grew on the Norway flower (i.e. Norway (small) flower x Chiltern pollen); this was closely followed by Chiltern pollen growing on the Chiltern flower (i.e. Chiltern (large) flower x Chiltern pollen) [see Fig. 5.2 and Tables 5.5]. Although, it is not very clear what could have affected the pollen-tube growth rate, it is obvious that both the pollen as well as the style environment have contributed to this pattern. It has been reported, however that the ability of pollen to compete is highly influenced by the recipient plants, and this could lead to inconsistent rank order of donors across recipients (Fenster and Sork, 1988; Cruzan, 1990; Johnston, 1993; and Marshall, 1998). Thus, this result corroborates the opinion expressed by Lankinen *et al* (2009), who noted that a pollen trait could be selected for even when there is female influence, provided that the trait confers a high average pollen competitive ability.

The results in this chapter have revealed that post-pollination selective mechanisms occur in populations of *Collinsia heterophylla*. The pollen-tube lengths (per unit time) for the Chiltern (large-flowered)

population were observed to show strong advantage over those of the Norway (small-flowered) population in both selfed and crossed pollination experiments. The Chiltern population is mainly large flowered and normally thought to be an outcrosser, while the Norway population is small-flowered and is presumably more inbreeding.

The advantage shown by Chiltern population over the Norway population indicates that if these two 'ecotypes' ever grew parapatrically, and the two types of pollen arrive on a stigma at the same time, the Chiltern pollen is likely to reach the ovary faster and hence, fertilises the ovule before the opportunity for the Norway pollen. It is believed that when this occurs between Chiltern and Norway populations, it will certainly have the advantage of increasing the vigour of the hybrid with the disadvantage of higher heterozygosity. Consequently, selection will favour genotypes from the Chiltern population and therefore evolution would occur if the differences have a genetic basis. Similar results have been reported from the study of *Nicotiana logiflora* (an outcrosser) by Figueroa-Castro (2009). The result in this chapter therefore confirms the report of several studies reviewed by Brandvain and Haig, (2005), where it was shown that parental conflict is less intense in self-pollinating plants than in outbreeders, because outbreeding plants are pollinated by numerous pollen donors that are not related to the seed parent, while a self-pollinating plant is pollinated by itself. Consequently, in crosses between plants with differing mating systems, outbreeding parents are expected to "overpower" selfing parents. This is referred to as the weak inbreeder/strong outbreeder (WISO) hypothesis. In contrast, Lankinen *et al* (2009) observed that cross-pollen did not perform better than self-pollen in the pistil of *C. heterophylla*. They proposed that self-pollen could sometimes perform better than outcross pollen, based on germination percentage on fully receptive stigmas. However, the result of Lankinen *et al* (2009) was based on the studies of a single population (i.e. the Southern California population via Norway).

Another important finding observed in this study is the pattern observed when selfed and inter-population cross-pollination treatments

were compared within populations. The results obtained showed that Norway pollen grew faster on the Chiltern flower than on the Norway flower. Similarly, the Chiltern pollen grew faster on the Norway flower than on the Chiltern flower. Although, *C. heterophylla* has been reported to be self-compatible (see Armbruster *et al.*, 2002; Lankinen *et al.*, 2009), the pattern of pollen-tube growth observed within populations has revealed that there is some cryptic self-incompatibility (CSI) taking place. Similar results have been documented in *Decodon verticilatus* by Allen and Eckert, (1997). According to Mazer and Travers (2000) cryptic self-incompatibility occurs as a result of superior pre-fertilization performance (pollen germination rate and pollen-tube growth rate) of outcross pollen relative to self pollen. This CSI has been suggested to have consequences for mating systems evolution, since CSI resulting from differential pollen-tube growth may minimize geitonogamous selfing when cross pollen is plentiful, while maximizing fecundity when cross pollen is limited (Eckert and Allen, 1997).

It is therefore evident that pollen germination and pollen-tube growth rates are not only influenced by the environment within the pollen grain but by the environment in the style through which it grows. This intra-style environmental factor has been suggested to be the mechanism of self-incompatibility, inbreeding depression, or outbreeding depression in some plant species (e.g. Fenster and Sork, 1988; Cruzan, 1990; Johnson, 1993; reviewed in Lankinen *et al.*, 2009). Even though *C. heterophylla* is a self-compatible annual; the influence of the stylar environment on pollen-tube germination and growth could determine the direction of the evolution of species. It was reported by Herrero and Hormaza (1996) that *Collinsia* pollen exploits both stored and exogenous resources comparable to uptake of nutrients from the pistil after initial germination and growth (see Lankinen *et al.*, 2009).

The overall result obtained in this chapter showed that pollen-tube growth rate on the style of the recipient flower varied between the populations studied and such variation is able to generate siring success and could influence the evolution of plant species (see Lankinen *et al.*,

2009). Therefore it is suggested that when different populations of *C. heterophylla* with varying flower sizes (large and small) grow together in the field under natural environmental conditions, the resulting progeny are more likely to be large flowered plant, provided there is abundant pollinator visitation. However, where pollinator visitation is unpredictable, the resulting progeny will most likely be a mixed population of small- and large-flowered plants. Therefore, both flower size and pollinator visitation rate are complementary factors in driving the evolution of mating system in *Collinsia heterophylla*.

5.6 Conclusion

The experiment in this chapter examined the behaviour of pollen from Chiltern (large-flowered) and Norway (small-flowered) populations of *C. heterophylla,* and the post-pollination mechanisms that could influence pollen-tube growth rate; and consequently, determine the course of the evolution of mating systems in *C. heterophylla*. It was apparent from the results obtained that germinating pollen in the growth medium is not a reliable assessment of pollen performance and therefore cannot be used to predict pollen performance on the recipient plant styles. There was no correlation in pollen-tube growth rate for *in-vitro* and *in-vivo* germination treatments within populations studied. Results obtained also showed that pollen from Chiltern population (large-flowered) *C. heterophylla* grew faster than pollen from the Norway (small-flowered) population, irrespective of the type of flower on the recipient plant. However, cryptic self-incompatibility (CSI) was observed in the two populations studied as Chiltern pollen were found to produce longer pollen-tubes on the Norway flower than on Chiltern flower; and Norway pollen produced longer pollen-tube on the Chiltern flower than on the Norway flower. But overall, Chiltern pollen were observed to have the longest pollen-tube growth *in-vivo*; suggesting that Chiltern (large-flowered) pollen has higher competitive ability and capable of over-powering the Norway (small-flowered) pollen. Therefore, post-pollination processes, such as, pollen-tube growth rate has a potential to influence the course of mating system

evolution in flowering plants. However, in order to affirm this result, it is suggested that further study be carried out using larger populations of *Collinsia* sp. Attempts should also be made in future studies to grow the two types of pollen on the same style using genetic markers (e.g. Simple Sequence Repeat markers) and observe the competitive ability of each type of pollen. In addition, the study of pollination and siring success of both pollen types (Norway and Chiltern) could provide complementary evidence.

CHAPTER 6

6.0 General Discussion and Conclusions

6.1 Introduction

In angiosperms, mating systems vary significantly within and among species, and are controlled by several classes of floral adaptations. These floral adaptations are the causes of morphological and structural variations in flowers. The structural variation in flowers and inflorescences vary greatly among populations and within species; as well as largely influence the quantity and quality of pollen dispersed during pollination. Also, these flower characteristics correlate with the mating systems adopted by each individual or population/species. In particular, the different mechanism adopted by each species for the positioning of male and female gametes is astonishing (Barrett, 1998). These structural variations in flowers have fascinated evolutionary biologists for over a century since the time of Darwin (1877, 1878), and careful studies of intra-specific variations, especially in wide-ranging species adapted to different ecological conditions, could often provide significant insights into variation and evolution of reproductive traits within families and genera (Barrett, 2008).

The research presented in this thesis has studied the intra-specific variations, correlations and heritabilities of floral morphological traits displayed by two populations of *C. heterophylla* adapted to different geographical locations; with the aim of unveiling how these floral-traits variations could drive the evolution of mating systems in this species. Previous research studies, for example, Armbruster *et al.* (2002) have suggested that *C. heterophylla* exhibits a mixed mating system rather than categorising it into distinct selfing or outcrossing. Species exhibiting mixed mating systems frequently demonstrate variable expression of breeding system characteristics and as a result represent the opportunity to understand the factors and mechanisms that promote both outcrossed

and selfed seed production (Chen *et al.*, 2009). Therefore, *C. heterophylla* is a good model plant for this study.

In this thesis, two populations of *C. heterophylla* having distinct flower sizes and obtained from two different geographical locations/ecological environments were studied. One population is mainly small-flowered (Norway population), and the second population is normally large-flowers (Chiltern population). Intra-specific variations in floral-traits were examined in these two populations for correlations, heritabilities, floral longevity and pollen-tube growth rate. The results obtained have shed more light on some of the unanswered questions on the evolution of mating systems in wild plant populations using the genus *Collinsia* and *Tonella* (see work done by Armbruster *et al.*, 2002; Kalisz *et al.*, 2004; Lankinen *et al.*, 2007; 2009).

6.1.1 Floral-Traits Variations, Correlations, and Heritabilities

One obvious characteristic that affects the mating systems of angiosperms is the flower size. In chapter three of this thesis, the two populations of *C. heterophylla* studied were examined for floral-traits variations. It was observed that although, floral-traits measured varied continuously in this study plant; the variations among plants were not significant but the variations between populations were highly significant, and this agrees with the results of most previous studies (e.g. Schwaegerle *et al.*, 1986; Herrera, 1990; Armbruster, 1991 and Dominguez et al., 1998. In addition significant floral-traits variations were observed across the five floral developmental stages within populations of *Collinsia heterophylla*. This result is consistent with the results obtained by Campbell (1992), who reported that definite changes occur from one floral developmental stage to the other in *Ipomopsis aggregata*, but that considerable variation was observed among plants. However, variations among plants in *C. heterophylla* were not significant, because flowers varied continuously among plants within- as well as between-populations. This was expected since floral-traits were measured at different floral developmental stages within- and among-populations. Besides,

Armbruster *et al.* (2002) have reported that floral-traits measured in the Southern California population of *C. heterophylla* showed continuous variation rather than falling into discrete groups.

Moreover, the results obtained in chapter three showed that there were high phenotypic correlations among floral-traits that influence mating systems in *C. heterophylla*. Phenotypic correlations were found to be generally higher in the Norway population than in the Chiltern population. In the Norway population, the eight floral traits measured were found to correlate with each other; some floral-traits were highly correlated (corolla length, keel length, stamen1 length, pistil length and stamen3 length), while the banner length and the ASC were moderately correlated. The Chiltern population showed similar results to the Norway population but the banner length in the Chiltern population displayed higher correlations with other floral-traits, and lower correlations with the ASC. This pattern of correlations suggests that there is a substantial quantitative genetic variability between-population, and it is consistent with the result obtained by Weil and Allard (1964), who observed a similar result in *C. heterophylla* populations studied. Likewise, van Kleunen and Ritland (2004) have reported positive correlations between floral sizes measured in *Mimulus guttatus*.

Furthermore, some of the floral-traits in the two populations studied recorded significantly higher values for phenotypic correlations than other traits, e.g. corolla length, keel length, stamens and pistils. These high phenotypic correlations could have two or more possible explanations. The first is that the stamens, pistil and the corolla tube may be more closely related developmentally, hence, the high phenotypic correlation may be due to a high degree of pleiotropy in the genes affecting these traits. Similar results have been obtained from other plant species showing that stamens and petals are more closely related in development than are the remaining floral parts (Hill and Lord, 1989; Conner and Via, 1993). However, Conner and Via (1993) have suggested that since phenotypic variation among these same floral-traits do not always show a high correlation in other species, the developmental relationship proposed may

not be common to all angiosperms. The second explanation given for the particularly high phenotypic correlations in floral-traits observed is that selection might have acted to increase the correlations for effective pollination (Conner and Via, 1993). Another possibility for these high correlations could be the result of linkage of genes that control these floral-traits development. According to Caruso (2006), strong genetic correlations would restrict independent evolution of floral traits (see also, Bissell and Diggle, 2008).

Results obtained for the genetic correlations of all the floral-traits measured in the two populations were very similar. The banner length and the wing width showed low correlations and coefficient of determination; this was more prominent in the Chiltern population. The reason for this trend is not well understood, but could be the result of environmental influence/sensitivity. Moreover, since the results of the phenotypic and genetic correlations in the banner length and wing width in the Norway population differ significantly from the Chiltern population, the variations observed could be attributed to some other factors in the environment other than genetic (maternal effect inclusive). Murren (2002), has observed that floral-traits correlations usually appear plastic, reflecting the environmental sensitivity (see also Schlichting, 1989; Waitt and Levin, 1993). This environmental sensitivity could lead to low repeatabilities in the wing width and banner length, and therefore less likely to respond to selection, but have a high potential for evolutionary modification (Mazer and Dawson, 2001; Parachnowitsch and Elle, 2004). Although, floral-traits are often less plastic than vegetative traits (Williams and Conner, 2001), the results obtained for banner length and wing width indicate some environmental effect (noise). However, the corolla, stamens and pistil were observed to have higher correlations and coefficient of determination (linear relationship). This suggests that they would respond more to selection as they seem to have been less affected by the environmental noises.

These phenotypic and genetic floral-traits variations are not unconnected with the mating strategy employed by each of the two

populations studied. Several studies have reported that small (reduced) flower size are associated with selfing species compared with large flower size commonly connected with outcrossing species (Ritland and Ritland, 1989; Dole, 1992; Schoen *et al.*, 1997; Eckhart and Geber, 1999; Armbruster *et al.*, 2002). Prior studies on *Collinsia heterophylla* have also reported that small-flowered species are generally selfing while the large-flowered species are mainly out-crossing, but that the floral-traits measured showed continuous variation rather than falling into discrete groups (Armbruster *et al.*, 2002).

In addition, chapter three of this thesis tested the ability of these floral-traits to evolve by estimating the heritabilities of the eight floral-traits measured using the parent-offspring regression. In this study, all eight floral-traits measured in the small-flowered (Norway) population showed positive high heritabilities; while in the large-flowered (Chiltern) population, only seven of the traits showed positive high heritabilities. The results obtained in the Norway population are similar to the results obtained by Lankinen *et al.* (2007) for the Norway population of *Collinsia heterophylla*. For example, heritabilities and correlations for ASC (h^2 = 0.777, r = 0.667, N = 60, P < 0.001) obtained in this study are very similar to those observed by Lankinen *et al.* (2007), who reported Pearson correlation - r = 0.668, N = 10, P = 0.035. The results of heritabilities showed that floral-traits measured are mainly influenced by genetic factors, and therefore will evolve. Van Kleunen and Ritland (2004) have earlier predicted that large flowers with large reproductive organs, which generally favour outcrossing, will evolve in natural populations of *Mimulus guttatus*.

However, in order to fully comprehend the evolution of mating systems in *C. heterophylla*, it is important to investigate floral-traits development from flower bud initiation through anthesis to senescence. Therefore, in chapter four of this thesis, flower longevity was assessed in the two populations of *C. heterophylla* studied. Several factors have been found to influence flower longevity, these include time of pollination and pollen source. Chen *et al.* (2009) have suggested that comprehensive

understanding of mating systems can only be achieved when both reproduction and pollination systems are studied within the context of each other. Consequently, the effect of pollination treatments on flower longevity was studied and compared within- and between-populations of *C. heterophylla*.

6.1.2 Pollination-Induced Flower Senescence

Flower longevity varies considerably among species of angiosperms. In many species flower longevity is partially dependent on pollination success (Devlin and Stephenson, 1984; Richardson and Stephenson, 1989; Stead, 1992; Proctor and Harder, 1995; Clayton and Aizen, 1996; VanDoorn, 1997; Yasaka *et al.*, 1998 reviewed in Sato, 2002). In chapter four of this thesis, floral longevity was assessed under four different pollination treatments as well as across four floral developmental stages (stages 0 – 3) in the two populations (Norway and Chiltern). The results obtained showed that pollination has the capacity to induced flower senescence in the two populations; therefore, flower longevity in both populations was shortened by pollination. This is consistent with the work of earlier researchers who reported that floral longevity can be affected by successful pollination (see Devlin and Stephenson, 1984; Richardson and Stephenson, 1989; Proctor and Harder, 1995; Clayton and Aizen, 1996; Yasaka *et al.*, 1998; Sato, 2002; Weber and Goodwillie, 2007). Also, increased intensity of pollination has been found to shorten flower life and, consequently, reduces the cost of floral maintenance in outcrossing species (Wyatt, 1984; Primack, 1985; Ritland and Ritland, 1989; Dole, 1992).

In addition, the two populations studied responded differently to each of the four pollination treatments under the same greenhouse conditions. They showed significant difference in floral longevity under three pollination treatments, but no significant difference was observed in the emasculated pollination treatment. Also flower longevity observed was significantly different across all floral developmental stages in all four pollination treatments. Generally, small-flowered (Norway) population

showed reduced floral longevity than the large-flowered (Chiltern) population under three pollination treatments. This result agrees with the result of Rogers (2006), who found that flowers have a limited life span and that floral longevity is species-specific and largely independent of environmental factors. But the emasculated pollination treatment lived longer than other pollination treatments and showed similar results in the two populations (Norway – mean = 9.2±0.82s.d and Chiltern –mean = 9.1±1.51s.d). This demonstrates that both types of flowers in *C. heterophylla* will live longer in the absence of pollination, thereby affirming that pollination will normally induce flower senescence (Weber and Goodwillie, 2007). In contrast, to this result however, Ichimura and Suto (1998) have reported that emasculated flowers senesced faster and that wounding of filaments was even much more effective in accelerating flower senescence in *Portulaca* hybrids. However, there was significant difference in floral longevity for emasculated pollination treatment across the four floral developmental stages; this was expected as these floral developmental stages occur over three to four days of anthesis.

The results obtained also showed that flowers in the Norway population (small-flowered) senesced earlier than flowers in the Chiltern population (large-flowered). The reduced flower size is usually expected to have an advantage over the large flower size, because it is expected to increase the ease of autonomous pollen transfer, if sexual parts are in contact at anthesis; while spatial proximity increases the effectiveness of delayed selfing mechanism in larger flowers (Dole, 1990; Kalisz *et al.*, 1999). This result therefore corroborates the findings of Armbruster *et al.* (2002) who reported that in *C. heterophylla*, small flowers are associated with autonomous selfing, while large flowers are associated cross pollination. Therefore, this result corroborates the result obtained by Primack (1985), who observed that species of plants that undergo autonomous selfing commonly have shorter-lived flowers than plant species of the same genus, species or family that undergo outcrossing (see also He *et al.*, 2005). He then suggested that selfing species may profit from pollinating faster and moving on to develop fruits, while an

outcrossing species allows flowers to remain open for a longer time and hence the likelihood of pollinator visitation, resulting in increased plant vigour (Weber and Goodwillie, 2007). Therefore, in late-selfing flowers, whenever cross-pollination is achieved, floral longevity and selfing rate is decreased by terminating flowering before autonomous self-pollination occurs. Hence, the ability or timing of autonomous self-fertilisation should largely influence both the selfing rate and floral longevity (Sato, 2002).

Besides, autonomous selfing (bagged -BG pollination treatment) was observed to shorten flower longevity in both populations of *Collinsia heterophylla*. This agrees with the result obtained by Weber and Goodwillie (2007), who showed that autonomous selfing reduces flower longevity in *Leptosiphon jepsonii,* and is also consistent with experiments reported on other plant species (see Underwood et al., 2005; Rogers, 2006). Autonomous selfing in the small-flowered (Norway) population occurred earlier than in the large-flowered (Chiltern) population, for example, autonomous selfing flower senescence in the Norway population (mean days = 4.22±1.47s.d), while in the Chiltern population (mean days = 7.76±1.15s.d). This shows that autonomous selfing in small flowers occurred prior, while late selfing is the rule in large flowers. This result therefore predicts that, in an outcrossing population (large-flowered), ovule fertilisation only triggers flower senescence after substantial amount of pollen have been dispersed. While in the selfing species (small-flowered), autonomous self-fertilisation of ovules that occurs early in anthesis may trigger flower senescence before any opportunity for dispersal of pollen by a vector. This prior and late selfing observed is thought to be connected to the different rates at which floral parts elongate in the two populations, as well as the time of stigma receptivity. Armbruster *et al* (2002) have reported that all large flowered *Collinsia* sp have delayed stigma receptivity while the small-flowered species have early/prior stigma receptivity. Consequently, selfing-induced flower senescence might be viewed as a potential source of pollen discounting, that is a reduction in male outcross success (Holsinger *et al.*, 1984; reviewed in Weber and Goodwillie, 2007).

Although the results in this chapter confirms that *C. heterophylla* is self-compatible (see also Armbruster *et al.*, 2002), and that autonomous self pollination can occur in the two types of flowers/populations studied; however, flowers senesced faster in the two populations with inter-population cross pollination treatment than with self pollination treatment. This reveals that pollen grains germinated and grew faster on the inter-population cross-styles than on self-styles, showing a form of partial cryptic self-incompatibility in this species. This type of partial cryptic self-incompatibility could have consequences on mating systems evolution in this species. This suggests that when pollinators are high, cross-pollination is realised early and can lead to decrease in floral longevity, as well as decreased selfing rates by ending flowering time before autonomous self fertilisation occurs (Sato, 2002). In contrast, it has been reported that, even though pollinators may be high in an area, the rate of self pollination could still be maintained; since pollinators are likely to distribute the pollen to other flowers on the same plant first before going to visit neighbouring plants, thereby causing extensive pollen dispersal among flowers of the same plant (Robertson, 1992; Harder and Barrett, 1985; Brunet and Eckert, 1998; Eckert, 2000; Montaner *et al.*, 2001; Williams *et al.*, 2001; Elle and Hare, 2002; Karron *et al.*, 2004).

Overall, the crossed, selfed and un-manipulated pollination treatments were significantly different within- and between-populations; but the crossed (HC) pollination treatment in the two populations recorded the lowest number of days to flower senescence. Therefore it is thought that aside from the partial cryptic self-incompatibility suggested above, another reason for the trend in the result could be differential pollen-tube growth rate between the two populations studied. According to Figueroa-Castro (2008) the abilities of pollen grains to germinate and develop pollen-tubes to convey the male gametes to fertilise the ovules vary between the self and outcross donors, and this can consequently can influence the evolution and breeding systems of the angiosperms. Moreover, several studies have reported that outcross pollen-tubes have an advantage over self pollen-tubes when growing in the style (see

Bateman, 1956; Bowman, 1987; Casper *et al.*, 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al.*, 1990; Cruzan and Barrett, 1993; Rigney *et al.*, 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006; reviewed in Figueroa-Castro and Holtsford, 2009). Therefore, the chapter five of this thesis was designed to investigate the pollen-tube growth rate in the two populations, and to assess the post-pollination mechanisms that could influence the evolution mating systems in this study species.

6.1.3 Pollen-tube Growth Rate

The type of pollen a plant receives (e.g., selfed versus outcrossed) is likely to modify the mating system of the plant significantly (Levri, 1998). In chapter five of this thesis, pollen grains from Norway and Chiltern populations were grown on the growth medium (*in-vitro*), as well as on the style (*in-vivo*) of the recipient plants in order to assess pollen performance (pollen-tube growth rate) in *C. heterophylla*. In general, populations studied showed enormous variation in pollen-tube growth rate *in-vivo* (in the style). But the results obtained in the *in-vitro* experiment were very similar and were not significantly different in the two populations. The results showed that pollen grains of C. *heterophylla* are sensitive to the environment where they grow (see also Lankinen, 2001), and *in-vitro* pollen germination is unlikely to provide an accurate reflection of the response of pollen grains in the style of the recipient plant. However, Lankinen et al (2009) noted that growing pollen in the growth medium could be important especially in species with delayed stigma receptivity, where other options of assessing pollen performance excluding maternal influence are more difficult.

The *in-vivo* pollen germination showed that pollen grains in the two populations, Norway (small-flowered) and Chiltern (large-flowered), behaved differently on the recipient plant. The error bar plot revealed that the *in-vivo* pollen-tube growth rate is distinctly different across the four types of crosses carried out. This variation in pollen-tube length for crosses performed within- and between-populations was statistically

significant. The pattern observed is thought to be the result of interactions between the specific pollen and the particular style on which it is deposited. It has been reported in species of angiosperms that pollen traits vary among donors and hence siring success also vary among these donors (see Snow and Spira, 1996; Skogsmyr and Lankinen, 1999; and Lankinen *et al.*, 2009). This result agrees with the result of Ruane and Donohue (2007), who found that the siring success does not only depend on the environment within the pollen grain but, also depends on the stylar environment. In addition, some early researchers have reported that the environment in which the pollen germinates and grows (i.e. recipient plant) has the ability to greatly influence pollen-tube growth rate and the ability of the pollen to compete hence leading to siring success (see Fenster and Sork, 1988; Cruzan, 1990; Johnston, 1993).

The result of the *in-vivo* germination treatments within- and between-populations of *C. heterophylla* studied (Norway and Chiltern) also revealed that pollen from the Chiltern (large-flowered and normally outbreeding) population developed longer pollen-tubes than pollen from the Norway (small-flowered and usually selfing) population. This result is consistent with the report of Brandvain and Haig (2005) who, in their review of divergent mating systems and parental conflicts as barriers to hybridization in plants, noted that several studies have suggested that parental conflicts are less intense in selfing plants than in outbreeders. Consequently, outbreeders have developed adaptations that enable their pollen to compete with other related or unrelated pollen in the wild; one of such adaptations is the faster and longer pollen-tube growth. Therefore, in cross pollination experiments between species or populations with differing mating systems, outbreeding parents are expected to "overpower" selfing parents. This was referred to as the weak inbreeder/strong outbreeder (WISO) hypothesis. Also because pollen-tube growth rate has been reported to correlate with pollen size and style length, it has been suggested that pollen-tube of long-styled species are expected to have advantage over pollen-tube of short-styled species (Travers and Mazer,

2000; Kruszewski and Galloway, 2006; Lee *et al.* 2008; Figueroa-Castro and Holtsford, 2009).

Furthermore, the pattern of results obtained within each population for the different crosses (self and inter-population cross) carried out *in-vivo* showed that pollen from a different population performed better than pollen from the same population on the style of the recipient plant. For example, it was observed that pollen from Norway population developed longer pollen tubes on the style of Chiltern (inter-population cross) flower than on the Norway (self) flower. Similar pattern was observed in the Chiltern population that is, pollen from Chiltern population developed longer pollen-tube on the Norway (inter-population cross) flower than on the Chiltern (self) flower. This result corroborates the report of Colling, et al. (2004) who observed that adding pollen from a different population of *Scorzonera humilis* strongly increased progeny fitness compared with both natural pollination and pollen supplement from the same population (see also, Holmes *et al.*, 2008; Bello-Bedoy and Núñez-Farfán, 2010).

Also the result obtained is consistent with earlier studies which reported that pollen from predominantly selfing population frequently perform less favourably than pollen from normally outcrossing population when growing on self style (and vice versa). This is referred to as cryptic self-incompatibility, that is, when physiological mechanisms acting in the pistil screen pollen receipt by rejecting certain male gametophyte, especially self pollen (Bertin and Sullivan, 1988; Kruszewski and Galloway, 2006). Although results have shown that *C. heterophylla* is self-compatible (see Armbruster *et al.*, 2002; Lankinen *et al.*, 2009), the pattern of pollen-tube growth observed within- and between-populations has indicated that some form of partial cryptic self-incompatibility (CSI) occurs in *C. heterophylla*. This is comparable to the results documented for *Decodon verticilatus* by Eckert and Allen, (1997). This CSI has been suggested to have consequences for mating system evolution, since CSI resulting from differential pollen-tube growth may minimize geitonogamous selfing when inter-population cross pollen is plentiful, while maximizing fecundity when inter-population cross pollen is limited

(Eckert and Allen, 1997). In addition, this result confirms the report of Herrero and Hormaza (1996) that pollen germination and pollen-tube growth rate in *C. heterophylla* is not only influenced by the environment within the pollen grain but also influenced by the environment in the style through which the pollen grows (see Lankinen *et al.*, 2009). This intra-style environmental factor has been suggested to be the source of self-incompatibility, inbreeding depression, or outbreeding depression in some plant species (e.g. Fenster and Sork, 1988; Cruzan, 1990; Johnson, 1993; reviewed in Lankinen *et al.*, 2009).

In chapter five pollen-tube growth rates in the growth medium (*in-vitro*) varied significantly from pollen-tube growth in the style (*in-vivo*). But the results showed that pollen from the Chiltern population performed better on both self- and inter-population cross/recipient plants than the pollen from the Norway population. Therefore, it is suggested that in a natural habitat where the two populations are growing parapatrically, provided there is abundant pollinator visitation selection and consequent evolution will favour the Chiltern (large-flowered) population. However, when pollinator visitation is erratic, the resulting progeny will most likely be a mixed population of Norway and Chiltern *C. heterophylla* plants.

6.2 Conclusions

Overall, the results obtained from this research on mating systems evolution in *Collinsia heterophylla*, has lead to four important conclusions. First, floral traits varied continuously in *C. heterophylla* but flowers can still be distinctly categorised into small and large flowers. This implies that, although a mixed mating system has been proposed for this genus, differential floral trait elongation and delayed stigma receptivity would classify populations into selfing or outcrossing. Secondly, floral traits associated with mating systems are highly correlated and heritable, therefore selection on one trait could lead to selection on the other; consequently leading to these traits evolving together. But because these correlated floral traits show similar results in both parent and offspring generations, it is suggested that the correlation among these traits is as a

result of pleiotropy or genetic linkage. Therefore, change in one trait is likely to influence how selection operates on other traits that are correlated to it. Thirdly, pollination and subsequent fertilisation of the ovules can induce flower senescence, thereby reducing floral longevity. This has significant effects on those traits that are genetically linked; since the death of the corolla terminates the function of the reproductive parts, except in cases where the ovary/ovules have been fertilised and subsequently grow into fruit/seeds. Hence, if a flower of *C. heterophylla* is not pollinated, it is likely to live longer on the parent plant before senescing; thereby prolonging the life span of all functionally related traits. However, inter-population cross pollination led to earlier flower senescence than autonomous self pollination; suggesting differential pollen-tube growth rate and some form of cryptic self-incompatibility. Fourthly, outcross pollen [from Chiltern (large-flowered) population] has a higher competitive ability than the self pollen [from Norway (small-flowered population], agreeing to the weak inbreeder/strong outcrosser (WISO) hypothesis; and therefore outcross (Chiltern) pollen will possibly mount a stronger challenge against the physiological barrier in the style of both self and outcross flowers than the self (Norway) pollen. Nevertheless, this competition is only effective and advantageous in a community where pollinators are present and the two ecotypes grow parapatrically. However, pollinator preference for one type of flower over the other may influence pollen dispersal and subsequently the mating system evolution of species in natural environment.

6.3 Future Research

In view of the fact that the evolution of quantitative traits (e.g. floral traits) depend on their heritability and evolvability, it will therefore be necessary to carry out a selection experiment both in the greenhouse and in the natural habitat of *C. heterophylla* to determine accurately the heritability and evolvability of floral-traits associated with mating systems. This will help in understanding better what factors and interacting mechanism will drive the evolution of wild plants e.g. *Collinsia*.

Furthermore, hybridisation experiment between the Norway (small-flowered) and the Chiltern (large-flowered) populations, followed by a selection experiment (including measurements of all fitness components in all generations) is suggested to investigate the ability of species to evolve. This is because hybridisation increases the genetic variation present; according to Falconer (1981), no matter how intense selection may be, evolutionary change cannot occur unless adequate genetic variation is present. Hence, all else being equal, evolvability is proportional to genetic variability (Feder, 2007).

Also, it has been reported that late pollination reduces inflorescence maturation, fruit maturation, seed initiation, seed abortion, and seed germination in other plant species (e.g. see Levri, 1998), it will be of interest to examine these parameters in future studies of pollination at different floral developmental stages in the selfed and outcrossed populations of *C. heterophylla* in order to determine at what floral developmental stage, during anthesis, is the effect optimum for all the parameters mentioned above, as these will reveal the quality and vigour of the pollen source as well as have consequences for mating system evolution. This is because siring success in plant does not only depend on the quantity of pollen, but also depends on the quality and source of pollen.

Finally, to assess and have a better understanding of differential pollen-tube growth rate between-populations, as well as pollen competitive ability between the small- and large-flowered populations, multiple-donor crosses need to be carried out on both self- and outcross-styles using a genetic marker. According to Muraya et al (2010), paternity can be determined in the progeny by using two diagnostic Simple Sequence Repeat (or Microsatellites) markers, and these microsatellites can be amplified for identification by the PCR process. This technique will make it possible to assess the competitive abilities of pollen donors, and also help to ascertain as well as partition the source of variation in pollen-tube growth rate (maternal or paternal).

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APPENDICES

Appendix 1.0 Homogeneity and Normality Tests for Chapter 3

Test of Normality of Data

Tests of Normality[b]

	Floral Dev. Stages	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Corolla Length	.000	.118	20	.200*	.943	20	.272
	Stage 1	.178	20	.095	.931	20	.164
	Stage 2	.086	20	.200*	.984	20	.976
	stage 3	.123	20	.200*	.958	20	.504
	Stage 4	.117	20	.200*	.959	20	.520
Wing width	.000	.134	20	.200*	.942	20	.264
	Stage 1	.195	20	.044	.825	20	.002
	Stage 2	.196	20	.042	.920	20	.097
	stage 3	.155	20	.200*	.950	20	.371
	Stage 4	.146	20	.200*	.927	20	.137
Banner Length	.000	.113	20	.200*	.966	20	.668
	Stage 1	.123	20	.200*	.971	20	.778
	Stage 2	.119	20	.200*	.985	20	.980
	stage 3	.099	20	.200*	.972	20	.798
	Stage 4	.102	20	.200*	.970	20	.750
Keel Length	.000	.118	20	.200*	.956	20	.463
	Stage 1	.099	20	.200*	.969	20	.738
	Stage 2	.122	20	.200*	.971	20	.771
	stage 3	.141	20	.200*	.936	20	.205
	Stage 4	.140	20	.200*	.960	20	.552
Stamen 1 Length	.000	.137	20	.200*	.946	20	.311
	Stage 1	.094	20	.200*	.956	20	.476
	Stage 2	.148	20	.200*	.957	20	.493
	stage 3	.147	20	.200*	.934	20	.186
	Stage 4	.157	20	.200*	.959	20	.528
Pistil Length	.000	.126	20	.200*	.930	20	.157
	Stage 1	.211	20	.020	.896	20	.035
	Stage 2	.149	20	.200*	.883	20	.020
	stage 3	.121	20	.200*	.952	20	.398
	Stage 4	.105	20	.200*	.961	20	.571
Stamen 3 Length	.000	.167	20	.146	.931	20	.160
	Stage 1	.186	20	.069	.931	20	.162
	Stage 2	.163	20	.172	.932	20	.169
	stage 3	.114	20	.200*	.972	20	.788
	Stage 4	.128	20	.200*	.963	20	.595

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

b. Anther Stigma Contact is constant when Floral Dev. Stages = Stage 4. It has been omitted.

Test of Homogeneity of Variance

Levene's Test of Equality of Error Variances[a]

	F	df1	df2	Sig.
Corolla Length	1.117	4	95	.353
Wing width	.163	4	95	.956
Banner Length	.233	4	95	.919
Keel Length	1.164	4	95	.332
Stamen 1 Length	4.031	4	95	.005
Pistil Length	1.695	4	95	.157
Stamen 3 Length	1.741	4	95	.147
Anther Stigma Contact	19.694	4	95	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Plant + stage

Appendix 1.1 General Linear Model Multivariate Test on Pooled Data for Norway and Chiltern Populations Parental Generations

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.961	557.250[a]	8.000	182.000	.000
	Wilks' Lambda	.039	557.250[a]	8.000	182.000	.000
	Hotelling's Trace	24.494	557.250[a]	8.000	182.000	.000
	Roy's Largest Root	24.494	557.250[a]	8.000	182.000	.000
plant	Pillai's Trace	.097	2.438[a]	8.000	182.000	.016
	Wilks' Lambda	.903	2.438[a]	8.000	182.000	.016
	Hotelling's Trace	.107	2.438[a]	8.000	182.000	.016
	Roy's Largest Root	.107	2.438[a]	8.000	182.000	.016
Pop	Pillai's Trace	.462	19.569[a]	8.000	182.000	.000
	Wilks' Lambda	.538	19.569[a]	8.000	182.000	.000
	Hotelling's Trace	.860	19.569[a]	8.000	182.000	.000
	Roy's Largest Root	.860	19.569[a]	8.000	182.000	.000
stage	Pillai's Trace	1.320	11.391	32.000	740.000	.000
	Wilks' Lambda	.052	25.870	32.000	672.778	.000
	Hotelling's Trace	11.425	64.445	32.000	722.000	.000
	Roy's Largest Root	10.826	250.345[b]	8.000	185.000	.000
Pop * stage	Pillai's Trace	.611	4.168	32.000	740.000	.000
	Wilks' Lambda	.496	4.398	32.000	672.778	.000
	Hotelling's Trace	.813	4.585	32.000	722.000	.000
	Roy's Largest Root	.471	10.890[b]	8.000	185.000	.000

a. Exact statistic
b. The statistic is an upper bound on F that yields a lower bound on the significance level.
c. Design: Intercept + plant + Pop + stage + Pop * stage

Appendix 1.2 General Linear Model Multivariate Test on Data for:

Norway Population Parental Generation

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.997	4057.377[a]	8.000	87.000	.000
	Wilks' Lambda	.003	4057.377[a]	8.000	87.000	.000
	Hotelling's Trace	373.092	4057.377[a]	8.000	87.000	.000
	Roy's Largest Root	373.092	4057.377[a]	8.000	87.000	.000
plant	Pillai's Trace	.005	.056[a]	8.000	87.000	1.000
	Wilks' Lambda	.995	.056[a]	8.000	87.000	1.000
	Hotelling's Trace	.005	.056[a]	8.000	87.000	1.000
	Roy's Largest Root	.005	.056[a]	8.000	87.000	1.000
stage	Pillai's Trace	1.853	9.712	32.000	360.000	.000
	Wilks' Lambda	.015	21.608	32.000	322.435	.000
	Hotelling's Trace	20.887	55.807	32.000	342.000	.000
	Roy's Largest Root	19.140	215.325[b]	8.000	90.000	.000

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept + plant + stage

Chiltern Population Parental Generation

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.984	661.047[a]	8.000	87.000	.000
	Wilks' Lambda	.016	661.047[a]	8.000	87.000	.000
	Hotelling's Trace	60.786	661.047[a]	8.000	87.000	.000
	Roy's Largest Root	60.786	661.047[a]	8.000	87.000	.000
plant	Pillai's Trace	.341	5.626[a]	8.000	87.000	.000
	Wilks' Lambda	.659	5.626[a]	8.000	87.000	.000
	Hotelling's Trace	.517	5.626[a]	8.000	87.000	.000
	Roy's Largest Root	.517	5.626[a]	8.000	87.000	.000
stage	Pillai's Trace	1.374	5.884	32.000	360.000	.000
	Wilks' Lambda	.041	13.855	32.000	322.435	.000
	Hotelling's Trace	14.236	38.036	32.000	342.000	.000
	Roy's Largest Root	13.650	153.563[b]	8.000	90.000	.000

a. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level.

c. Design: Intercept + plant + stage

Appendix 1.3 General Linear Model Multivariate ANOVA on Pooled Data for Norway and Chiltern Populations Parental Generations

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
plant	corl	.577	1	.577	.397	.530
	wingw	2.215	1	2.215	1.640	.202
	bannerl	1.267	1	1.267	2.122	.147
	keell	3.675	1	3.675	2.636	.106
	stamen1l	1.948	1	1.948	1.849	.176
	pistill	.175	1	.175	.144	.705
	stamen3l	2.742	1	2.742	2.386	.124
	ASC	7.368E-5	1	7.368E-5	.001	.973
Pop	corl	155.896	1	155.896	107.157	.000
	wingw	190.944	1	190.944	141.434	.000
	bannerl	45.435	1	45.435	76.082	.000
	keell	134.668	1	134.668	96.597	.000
	stamen1l	54.148	1	54.148	51.398	.000
	pistill	29.324	1	29.324	24.187	.000
	stamen3l	24.665	1	24.665	21.457	.000
	ASC	.105	1	.105	1.671	.198
stage	corl	347.132	4	86.783	59.651	.000
	wingw	139.111	4	34.778	25.760	.000
	bannerl	20.883	4	5.221	8.742	.000
	keell	296.376	4	74.094	53.147	.000
	stamen1l	515.489	4	128.872	122.328	.000
	pistill	1108.931	4	277.233	228.675	.000
	stamen3l	1095.148	4	273.787	238.183	.000
	ASC	27.759	4	6.940	110.092	.000
Pop * stage	corl	4.805	4	1.201	.826	.510
	wingw	1.476	4	.369	.273	.895
	bannerl	.195	4	.049	.082	.988
	keell	1.458	4	.365	.262	.902
	stamen1l	6.971	4	1.743	1.654	.162
	pistill	30.241	4	7.560	6.236	.000
	stamen3l	21.441	4	5.360	4.663	.001
	ASC	2.419	4	.605	9.594	.000
Error	corl	274.964	189	1.455		
	wingw	255.161	189	1.350		
	bannerl	112.869	189	.597		
	keell	263.490	189	1.394		
	stamen1l	199.111	189	1.053		
	pistill	229.133	189	1.212		
	stamen3l	217.252	189	1.149		
	ASC	11.914	189	.063		
Total	corl	75033.314	200			
	wingw	22941.260	200			
	bannerl	10864.784	200			
	keell	63949.261	200			
	stamen1l	47749.904	200			
	pistill	35724.412	200			
	stamen3l	35178.406	200			
	ASC	79.000	200			
Corrected Total	corl	1188.116	199			
	wingw	1027.053	199			
	bannerl	268.427	199			
	keell	961.274	199			
	stamen1l	875.064	199			
	pistill	1470.804	199			
	stamen3l	1386.571	199			
	ASC	42.534	199			

a. R Squared = .769 (Adjusted R Squared = .756)
b. R Squared = .752 (Adjusted R Squared = .738)
c. R Squared = .580 (Adjusted R Squared = .557)
d. R Squared = .726 (Adjusted R Squared = .711)
e. R Squared = .772 (Adjusted R Squared = .760)
f. R Squared = .844 (Adjusted R Squared = .836)

g. R Squared = .843 (Adjusted R Squared = .835)
h. R Squared = .720 (Adjusted R Squared = .705)

Appendix 1.4 General Linear Model Multivariate ANOVA on Data for Norway Population Parental Generation

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	corl	143.430[a]	5	28.686	67.965	.000
	wingw	58.672[b]	5	11.734	26.428	.000
	bannerl	12.109[c]	5	2.422	13.507	.000
	keell	142.027[d]	5	28.405	61.507	.000
	stamen1l	241.509[e]	5	48.302	89.508	.000
	pistill	460.009[f]	5	92.002	235.416	.000
	stamen3l	583.782[g]	5	116.756	88.097	.000
	ASC	11.699[h]	5	2.340	28.266	.000
Intercept	corl	7117.028	1	7117.028	16862.306	.000
	wingw	1740.627	1	1740.627	3920.165	.000
	bannerl	955.628	1	955.628	5329.558	.000
	keell	6214.131	1	6214.131	13455.663	.000
	stamen1l	4855.866	1	4855.866	8998.371	.000
	pistill	3568.507	1	3568.507	9131.164	.000
	stamen3l	3611.765	1	3611.765	2725.201	.000
	ASC	3.084	1	3.084	37.260	.000
plant	corl	.007	1	.007	.016	.899
	wingw	.022	1	.022	.049	.826
	bannerl	.050	1	.050	.278	.599
	keell	.042	1	.042	.092	.762
	stamen1l	.043	1	.043	.079	.779
	pistill	.044	1	.044	.114	.737
	stamen3l	.005	1	.005	.004	.949
	ASC	.007	1	.007	.084	.773
stage	corl	143.423	4	35.856	84.953	.000
	wingw	58.650	4	14.663	33.022	.000
	bannerl	12.060	4	3.015	16.814	.000
	keell	141.985	4	35.496	76.861	.000
	stamen1l	241.466	4	60.366	111.865	.000
	pistill	459.965	4	114.991	294.242	.000
	stamen3l	583.776	4	145.944	110.120	.000
	ASC	11.692	4	2.923	35.312	.000
Error	corl	39.674	94	.422		
	wingw	41.738	94	.444		
	bannerl	16.855	94	.179		
	keell	43.411	94	.462		
	stamen1l	50.726	94	.540		
	pistill	36.736	94	.391		
	stamen3l	124.580	94	1.325		
	ASC	7.781	94	.083		
Total	corl	30951.708	100			
	wingw	7659.106	100			
	bannerl	4205.654	100			
	keell	26881.516	100			
	stamen1l	21140.157	100			
	pistill	15802.549	100			
	stamen3l	16329.577	100			
	ASC	33.920	100			
Corrected Total	corl	183.104	99			
	wingw	100.410	99			
	bannerl	28.964	99			
	keell	185.439	99			
	stamen1l	292.235	99			
	pistill	496.745	99			
	stamen3l	708.362	99			
	ASC	19.480	99			

a. R Squared = .783 (Adjusted R Squared = .772)
b. R Squared = .584 (Adjusted R Squared = .562)
c. R Squared = .418 (Adjusted R Squared = .387)

d. R Squared = .766 (Adjusted R Squared = .753)
e. R Squared = .826 (Adjusted R Squared = .817)
f. R Squared = .926 (Adjusted R Squared = .922)
g. R Squared = .824 (Adjusted R Squared = .815)
h. R Squared = .601 (Adjusted R Squared = .579)

Appendix 1.5 General Linear Model Multivariate ANOVA on Data for Chiltern Population Parental Generation

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	corl	209.853[a]	5	41.971	16.823	.000
	wingw	87.008[b]	5	17.402	7.769	.000
	bannerl	12.313[c]	5	2.463	2.464	.038
	keell	162.124[d]	5	32.425	14.018	.000
	stamen1l	284.115[e]	5	56.823	36.294	.000
	pistill	679.352[f]	5	135.870	66.387	.000
	stamen3l	538.648[g]	5	107.730	113.055	.000
	ASC	18.491[h]	5	3.698	84.359	.000
Intercept	corl	10316.088	1	10316.088	4134.871	.000
	wingw	3708.530	1	3708.530	1655.706	.000
	bannerl	1644.874	1	1644.874	1645.987	.000
	keell	8910.606	1	8910.606	3852.144	.000
	stamen1l	6309.375	1	6309.375	4029.945	.000
	pistill	4458.459	1	4458.459	2178.441	.000
	stamen3l	4501.494	1	4501.494	4724.004	.000
	ASC	5.494	1	5.494	125.324	.000
plant	corl	1.339	1	1.339	.537	.466
	wingw	5.070	1	5.070	2.264	.136
	bannerl	3.295	1	3.295	3.297	.073
	keell	6.274	1	6.274	2.712	.103
	stamen1l	3.122	1	3.122	1.994	.161
	pistill	.145	1	.145	.071	.791
	stamen3l	5.836	1	5.836	6.124	.015
	ASC	.005	1	.005	.115	.735
stage	corl	208.514	4	52.128	20.894	.000
	wingw	81.938	4	20.484	9.145	.000
	bannerl	9.019	4	2.255	2.256	.069
	keell	155.850	4	38.962	16.844	.000
	stamen1l	280.994	4	70.248	44.869	.000
	pistill	679.207	4	169.802	82.967	.000
	stamen3l	532.813	4	133.203	139.787	.000
	ASC	18.486	4	4.622	105.420	.000
Error	corl	234.521	94	2.495		
	wingw	210.546	94	2.240		
	bannerl	93.936	94	.999		
	keell	217.437	94	2.313		
	stamen1l	147.169	94	1.566		
	pistill	192.383	94	2.047		
	stamen3l	89.572	94	.953		
	ASC	4.121	94	.044		
Total	corl	44081.606	100			
	wingw	15282.153	100			
	bannerl	6659.131	100			
	keell	37067.745	100			
	stamen1l	26609.747	100			
	pistill	19921.862	100			
	stamen3l	18848.829	100			
	ASC	45.080	100			
Corrected Total	corl	444.374	99			
	wingw	297.554	99			
	bannerl	106.250	99			
	keell	379.561	99			
	stamen1l	431.284	99			
	pistill	871.735	99			
	stamen3l	628.221	99			

	ASC	22.612	99			

a. R Squared = .472 (Adjusted R Squared = .444)
b. R Squared = .292 (Adjusted R Squared = .255)
c. R Squared = .116 (Adjusted R Squared = .069)
d. R Squared = .427 (Adjusted R Squared = .397)
e. R Squared = .659 (Adjusted R Squared = .641)
f. R Squared = .779 (Adjusted R Squared = .768)
g. R Squared = .857 (Adjusted R Squared = .850)
h. R Squared = .818 (Adjusted R Squared = .808)

Appendix 1.6 Post Hoc Tests for Norway Population Parental Generation

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) stage	(J) stage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
corl	.00	1.00	-2.00644067*	.20437638	.000	-2.5747837	-1.4380976
		2.00	-2.58249067*	.20437638	.000	-3.1508337	-2.0141476
		3.00	-2.87358234*	.20437638	.000	-3.4419254	-2.3052393
		4.00	-3.50151434*	.20437638	.000	-4.0698574	-2.9331713
	1.00	.00	2.00644067*	.20437638	.000	1.4380976	2.5747837
		2.00	-.57605000*	.20437638	.045	-1.1443930	-.0077070
		3.00	-.86714167*	.20437638	.000	-1.4354847	-.2987986
		4.00	-1.49507367*	.20437638	.000	-2.0634167	-.9267306
	2.00	.00	2.58249067*	.20437638	.000	2.0141476	3.1508337
		1.00	.57605000*	.20437638	.045	.0077070	1.1443930
		3.00	-.29109167	.20437638	.614	-.8594347	.2772514
		4.00	-.91902367*	.20437638	.000	-1.4873667	-.3506806
	3.00	.00	2.87358234*	.20437638	.000	2.3052393	3.4419254
		1.00	.86714167*	.20437638	.000	.2987986	1.4354847
		2.00	.29109167	.20437638	.614	-.2772514	.8594347
		4.00	-.62793200*	.20437638	.023	-1.1962750	-.0595890
	4.00	.00	3.50151434*	.20437638	.000	2.9331713	4.0698574
		1.00	1.49507367*	.20437638	.000	.9267306	2.0634167
		2.00	.91902367*	.20437638	.000	.3506806	1.4873667
		3.00	.62793200*	.20437638	.023	.0595890	1.1962750
wingw	.00	1.00	-1.25841990*	.20965991	.000	-1.8414557	-.6753841
		2.00	-1.63454707*	.20965991	.000	-2.2175829	-1.0515113
		3.00	-1.99406957*	.20965991	.000	-2.5771054	-1.4110338
		4.00	-2.13800923*	.20965991	.000	-2.7210450	-1.5549734
	1.00	.00	1.25841990*	.20965991	.000	.6753841	1.8414557
		2.00	-.37612717	.20965991	.383	-.9591630	.2069086
		3.00	-.73564967*	.20965991	.006	-1.3186855	-.1526139
		4.00	-.87958933*	.20965991	.001	-1.4626251	-.2965535
	2.00	.00	1.63454707*	.20965991	.000	1.0515113	2.2175829
		1.00	.37612717	.20965991	.383	-.2069086	.9591630
		3.00	-.35952250	.20965991	.430	-.9425583	.2235133
		4.00	-.50346217	.20965991	.124	-1.0864980	.0795736
	3.00	.00	1.99406957*	.20965991	.000	1.4110338	2.5771054
		1.00	.73564967*	.20965991	.006	.1526139	1.3186855
		2.00	.35952250	.20965991	.430	-.2235133	.9425583
		4.00	-.14393967	.20965991	.959	-.7269755	.4390961
	4.00	.00	2.13800923*	.20965991	.000	1.5549734	2.7210450
		1.00	.87958933*	.20965991	.001	.2965535	1.4626251
		2.00	.50346217	.20965991	.124	-.0795736	1.0864980
		3.00	.14393967	.20965991	.959	-.4390961	.7269755
bannerl	.00	1.00	-.48328533*	.13339594	.004	-.8542414	-.1123293
		2.00	-.69163400*	.13339594	.000	-1.0625900	-.3206780
		3.00	-.89731733*	.13339594	.000	-1.2682734	-.5263613
		4.00	-.96445567*	.13339594	.000	-1.3354117	-.5934996
	1.00	.00	.48328533*	.13339594	.004	.1123293	.8542414
		2.00	-.20834867	.13339594	.525	-.5793047	.1626074

		3.00	-.41403200*	.13339594	.021	-.7849880	-.0430760
		4.00	-.48117033*	.13339594	.004	-.8521264	-.1102143
	2.00	.00	.69163400*	.13339594	.000	.3206780	1.0625900
		1.00	.20834867	.13339594	.525	-.1626074	.5793047
		3.00	-.20568333	.13339594	.538	-.5766394	.1652727
		4.00	-.27282167	.13339594	.253	-.6437777	.0981344
	3.00	.00	.89731733*	.13339594	.000	.5263613	1.2682734
		1.00	.41403200*	.13339594	.021	.0430760	.7849880
		2.00	.20568333	.13339594	.538	-.1652727	.5766394
		4.00	-.06713833	.13339594	.987	-.4380944	.3038177
	4.00	.00	.96445567*	.13339594	.000	.5934996	1.3354117
		1.00	.48117033*	.13339594	.004	.1102143	.8521264
		2.00	.27282167	.13339594	.253	-.0981344	.6437777
		3.00	.06713833	.13339594	.987	-.3038177	.4380944
keell	.00	1.00	-1.79560200*	.21387118	.000	-2.3903488	-1.2008552
		2.00	-2.33791533*	.21387118	.000	-2.9326621	-1.7431686
		3.00	-2.89715350*	.21387118	.000	-3.4919003	-2.3024067
		4.00	-3.48005200*	.21387118	.000	-4.0747988	-2.8853052
	1.00	.00	1.79560200*	.21387118	.000	1.2008552	2.3903488
		2.00	-.54231333	.21387118	.091	-1.1370601	.0524334
		3.00	-1.10155150*	.21387118	.000	-1.6962983	-.5068047
		4.00	-1.68445000*	.21387118	.000	-2.2791968	-1.0897032
	2.00	.00	2.33791533*	.21387118	.000	1.7431686	2.9326621
		1.00	.54231333	.21387118	.091	-.0524334	1.1370601
		3.00	-.55923817	.21387118	.076	-1.1539849	.0355086
		4.00	-1.14213667*	.21387118	.000	-1.7368834	-.5473899
	3.00	.00	2.89715350*	.21387118	.000	2.3024067	3.4919003
		1.00	1.10155150*	.21387118	.000	.5068047	1.6962983
		2.00	.55923817	.21387118	.076	-.0355086	1.1539849
		4.00	-.58289850	.21387118	.058	-1.1776453	.0118483
	4.00	.00	3.48005200*	.21387118	.000	2.8853052	4.0747988
		1.00	1.68445000*	.21387118	.000	1.0897032	2.2791968
		2.00	1.14213667*	.21387118	.000	.5473899	1.7368834
		3.00	.58289850	.21387118	.058	-.0118483	1.1776453
stamen1l	.00	1.00	-2.91683033*	.23117283	.000	-3.5596907	-2.2739700
		2.00	-3.31792217*	.23117283	.000	-3.9607825	-2.6750618
		3.00	-3.80964733*	.23117283	.000	-4.4525077	-3.1667870
		4.00	-4.53157972*	.23117283	.000	-5.1744400	-3.8887194
	1.00	.00	2.91683033*	.23117283	.000	2.2739700	3.5596907
		2.00	-.40109184	.23117283	.418	-1.0439522	.2417685
		3.00	-.89281700*	.23117283	.002	-1.5356773	-.2499567
		4.00	-1.61474939*	.23117283	.000	-2.2576097	-.9718891
	2.00	.00	3.31792217*	.23117283	.000	2.6750618	3.9607825
		1.00	.40109184	.23117283	.418	-.2417685	1.0439522
		3.00	-.49172517	.23117283	.217	-1.1345855	.1511352
		4.00	-1.21365755*	.23117283	.000	-1.8565179	-.5707972
	3.00	.00	3.80964733*	.23117283	.000	3.1667870	4.4525077
		1.00	.89281700*	.23117283	.002	.2499567	1.5356773
		2.00	.49172517	.23117283	.217	-.1511352	1.1345855
		4.00	-.72193238*	.23117283	.020	-1.3647927	-.0790721
	4.00	.00	4.53157972*	.23117283	.000	3.8887194	5.1744400
		1.00	1.61474939*	.23117283	.000	.9718891	2.2576097
		2.00	1.21365755*	.23117283	.000	.5707972	1.8565179
		3.00	.72193238*	.23117283	.020	.0790721	1.3647927
pistill	.00	1.00	-2.85688267*	.19676361	.000	-3.4040556	-2.3097097
		2.00	-3.64841600*	.19676361	.000	-4.1955890	-3.1012430
		3.00	-5.23317433*	.19676361	.000	-5.7803473	-4.6860014
		4.00	-6.21092233*	.19676361	.000	-6.7580953	-5.6637494
	1.00	.00	2.85688267*	.19676361	.000	2.3097097	3.4040556
		2.00	-.79153333*	.19676361	.001	-1.3387063	-.2443604
		3.00	-2.37629167*	.19676361	.000	-2.9234646	-1.8291187

		4.00	-3.35403967*	.19676361	.000	-3.9012126	-2.8068667
	2.00	.00	3.64841600*	.19676361	.000	3.1012430	4.1955890
		1.00	.79153333*	.19676361	.001	.2443604	1.3387063
		3.00	-1.58475834*	.19676361	.000	-2.1319313	-1.0375854
		4.00	-2.56250633*	.19676361	.000	-3.1096793	-2.0153334
	3.00	.00	5.23317433*	.19676361	.000	4.6860014	5.7803473
		1.00	2.37629167*	.19676361	.000	1.8291187	2.9234646
		2.00	1.58475834*	.19676361	.000	1.0375854	2.1319313
		4.00	-.97774800*	.19676361	.000	-1.5249210	-.4305750
	4.00	.00	6.21092233*	.19676361	.000	5.6637494	6.7580953
		1.00	3.35403967*	.19676361	.000	2.8068667	3.9012126
		2.00	2.56250633*	.19676361	.000	2.0153334	3.1096793
		3.00	.97774800*	.19676361	.000	.4305750	1.5249210
stamen3l	.00	1.00	-2.26468367*	.36213633	.000	-3.2717358	-1.2576316
		2.00	-3.17491793*	.36213633	.000	-4.1819700	-2.1678658
		3.00	-4.75983700*	.36213633	.000	-5.7668891	-3.7527849
		4.00	-7.20429284*	.36213633	.000	-8.2113449	-6.1972407
	1.00	.00	2.26468367*	.36213633	.000	1.2576316	3.2717358
		2.00	-.91023427	.36213633	.096	-1.9172864	.0968178
		3.00	-2.49515333*	.36213633	.000	-3.5022054	-1.4881012
		4.00	-4.93960917*	.36213633	.000	-5.9466613	-3.9325571
	2.00	.00	3.17491793*	.36213633	.000	2.1678658	4.1819700
		1.00	.91023427	.36213633	.096	-.0968178	1.9172864
		3.00	-1.58491906*	.36213633	.000	-2.5919712	-.5778670
		4.00	-4.02937490*	.36213633	.000	-5.0364270	-3.0223228
	3.00	.00	4.75983700*	.36213633	.000	3.7527849	5.7668891
		1.00	2.49515333*	.36213633	.000	1.4881012	3.5022054
		2.00	1.58491906*	.36213633	.000	.5778670	2.5919712
		4.00	-2.44445584*	.36213633	.000	-3.4515079	-1.4374037
	4.00	.00	7.20429284*	.36213633	.000	6.1972407	8.2113449
		1.00	4.93960917*	.36213633	.000	3.9325571	5.9466613
		2.00	4.02937490*	.36213633	.000	3.0223228	5.0364270
		3.00	2.44445584*	.36213633	.000	1.4374037	3.4515079
ASC	.00	1.00	-.17000	.09054	.336	-.4218	.0818
		2.00	-.29000*	.09054	.016	-.5418	-.0382
		3.00	-.44000*	.09054	.000	-.6918	-.1882
		4.00	-1.00000*	.09054	.000	-1.2518	-.7482
	1.00	.00	.17000	.09054	.336	-.0818	.4218
		2.00	-.12000	.09054	.676	-.3718	.1318
		3.00	-.27000*	.09054	.029	-.5218	-.0182
		4.00	-.83000*	.09054	.000	-1.0818	-.5782
	2.00	.00	.29000*	.09054	.016	.0382	.5418
		1.00	.12000	.09054	.676	-.1318	.3718
		3.00	-.15000	.09054	.466	-.4018	.1018
		4.00	-.71000*	.09054	.000	-.9618	-.4582
	3.00	.00	.44000*	.09054	.000	.1882	.6918
		1.00	.27000*	.09054	.029	.0182	.5218
		2.00	.15000	.09054	.466	-.1018	.4018
		4.00	-.56000*	.09054	.000	-.8118	-.3082
	4.00	.00	1.00000*	.09054	.000	.7482	1.2518
		1.00	.83000*	.09054	.000	.5782	1.0818
		2.00	.71000*	.09054	.000	.4582	.9618
		3.00	.56000*	.09054	.000	.3082	.8118

*. The mean difference is significant at the 0.05 level.

Appendix 1.7 Post Hoc Tests for Chiltern Population Parental Generation

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) stage	(J) stage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
corl	.00	1.00	-1.87466667*	.49827046	.003	-3.2602893	-.4890440
		2.00	-3.06983334*	.49827046	.000	-4.4554560	-1.6842107
		3.00	-3.46910000*	.49827046	.000	-4.8547227	-2.0834773
		4.00	-4.08770000*	.49827046	.000	-5.4733227	-2.7020773
	1.00	.00	1.87466667*	.49827046	.003	.4890440	3.2602893
		2.00	-1.19516667	.49827046	.125	-2.5807893	.1904560
		3.00	-1.59443333*	.49827046	.016	-2.9800560	-.2088107
		4.00	-2.21303333*	.49827046	.000	-3.5986560	-.8274107
	2.00	.00	3.06983334*	.49827046	.000	1.6842107	4.4554560
		1.00	1.19516667	.49827046	.125	-.1904560	2.5807893
		3.00	-.39926667	.49827046	.930	-1.7848893	.9863560
		4.00	-1.01786667	.49827046	.254	-2.4034893	.3677560
	3.00	.00	3.46910000*	.49827046	.000	2.0834773	4.8547227
		1.00	1.59443333*	.49827046	.016	.2088107	2.9800560
		2.00	.39926667	.49827046	.930	-.9863560	1.7848893
		4.00	-.61860000	.49827046	.727	-2.0042227	.7670227
	4.00	.00	4.08770000*	.49827046	.000	2.7020773	5.4733227
		1.00	2.21303333*	.49827046	.000	.8274107	3.5986560
		2.00	1.01786667	.49827046	.254	-.3677560	2.4034893
		3.00	.61860000	.49827046	.727	-.7670227	2.0042227
wingw	.00	1.00	-1.26896667	.47640751	.067	-2.5937914	.0558581
		2.00	-2.05066667*	.47640751	.000	-3.3754914	-.7258419
		3.00	-2.28643333*	.47640751	.000	-3.6112581	-.9616086
		4.00	-2.46840000*	.47640751	.000	-3.7932248	-1.1435752
	1.00	.00	1.26896667	.47640751	.067	-.0558581	2.5937914
		2.00	-.78170000	.47640751	.475	-2.1065248	.5431248
		3.00	-1.01746667	.47640751	.214	-2.3422914	.3073581
		4.00	-1.19943334	.47640751	.095	-2.5242581	.1253914
	2.00	.00	2.05066667*	.47640751	.000	.7258419	3.3754914
		1.00	.78170000	.47640751	.475	-.5431248	2.1065248
		3.00	-.23576667	.47640751	.988	-1.5605914	1.0890581
		4.00	-.41773333	.47640751	.905	-1.7425581	.9070914
	3.00	.00	2.28643333*	.47640751	.000	.9616086	3.6112581
		1.00	1.01746667	.47640751	.214	-.3073581	2.3422914
		2.00	.23576667	.47640751	.988	-1.0890581	1.5605914
		4.00	-.18196667	.47640751	.995	-1.5067914	1.1428581
	4.00	.00	2.46840000*	.47640751	.000	1.1435752	3.7932248
		1.00	1.19943334	.47640751	.095	-.1253914	2.5242581
		2.00	.41773333	.47640751	.905	-.9070914	1.7425581
		3.00	.18196667	.47640751	.995	-1.1428581	1.5067914
bannerl	.00	1.00	-.50313333	.31991986	.518	-1.3927871	.3865205
		2.00	-.66873333	.31991986	.233	-1.5583871	.2209205
		3.00	-.77750000	.31991986	.116	-1.6671538	.1121538
		4.00	-.83390000	.31991986	.077	-1.7235538	.0557538
	1.00	.00	.50313333	.31991986	.518	-.3865205	1.3927871
		2.00	-.16560000	.31991986	.985	-1.0552538	.7240538
		3.00	-.27436667	.31991986	.911	-1.1640205	.6152871
		4.00	-.33076667	.31991986	.839	-1.2204205	.5588871
	2.00	.00	.66873333	.31991986	.233	-.2209205	1.5583871
		1.00	.16560000	.31991986	.985	-.7240538	1.0552538
		3.00	-.10876667	.31991986	.997	-.9984205	.7808871
		4.00	-.16516667	.31991986	.986	-1.0548205	.7244871
	3.00	.00	.77750000	.31991986	.116	-.1121538	1.6671538
		1.00	.27436667	.31991986	.911	-.6152871	1.1640205

		2.00	.10876667	.31991986	.997	-.7808871	.9984205
		4.00	-.05640000	.31991986	1.000	-.9460538	.8332538
	4.00	.00	.83390000	.31991986	.077	-.0557538	1.7235538
		1.00	.33076667	.31991986	.839	-.5588871	1.2204205
		2.00	.16516667	.31991986	.986	-.7244871	1.0548205
		3.00	.05640000	.31991986	1.000	-.8332538	.9460538
keell	.00	1.00	-1.49333333*	.48526814	.022	-2.8427983	-.1438684
		2.00	-2.55700000*	.48526814	.000	-3.9064650	-1.2075350
		3.00	-2.93503334*	.48526814	.000	-4.2844983	-1.5855684
		4.00	-3.55210000*	.48526814	.000	-4.9015650	-2.2026350
	1.00	.00	1.49333333*	.48526814	.022	.1438684	2.8427983
		2.00	-1.06366667	.48526814	.192	-2.4131316	.2857983
		3.00	-1.44170000*	.48526814	.030	-2.7911650	-.0922350
		4.00	-2.05876667*	.48526814	.000	-3.4082316	-.7093017
	2.00	.00	2.55700000*	.48526814	.000	1.2075350	3.9064650
		1.00	1.06366667	.48526814	.192	-.2857983	2.4131316
		3.00	-.37803333	.48526814	.936	-1.7274983	.9714316
		4.00	-.99510000	.48526814	.250	-2.3445650	.3543650
	3.00	.00	2.93503334*	.48526814	.000	1.5855684	4.2844983
		1.00	1.44170000*	.48526814	.030	.0922350	2.7911650
		2.00	.37803333	.48526814	.936	-.9714316	1.7274983
		4.00	-.61706667	.48526814	.709	-1.9665316	.7323983
	4.00	.00	3.55210000*	.48526814	.000	2.2026350	4.9015650
		1.00	2.05876667*	.48526814	.000	.7093017	3.4082316
		2.00	.99510000	.48526814	.250	-.3543650	2.3445650
		3.00	.61706667	.48526814	.709	-.7323983	1.9665316
stamen11	.00	1.00	-2.14690000*	.39774415	.000	-3.2529726	-1.0408274
		2.00	-3.29283333*	.39774415	.000	-4.3989059	-2.1867607
		3.00	-3.96656667*	.39774415	.000	-5.0726393	-2.8604941
		4.00	-4.84750000*	.39774415	.000	-5.9535726	-3.7414274
	1.00	.00	2.14690000*	.39774415	.000	1.0408274	3.2529726
		2.00	-1.14593333*	.39774415	.038	-2.2520059	-.0398607
		3.00	-1.81966667*	.39774415	.000	-2.9257393	-.7135941
		4.00	-2.70060000*	.39774415	.000	-3.8066726	-1.5945274
	2.00	.00	3.29283333*	.39774415	.000	2.1867607	4.3989059
		1.00	1.14593333*	.39774415	.038	.0398607	2.2520059
		3.00	-.67373333	.39774415	.443	-1.7798059	.4323393
		4.00	-1.55466666*	.39774415	.002	-2.6607393	-.4485941
	3.00	.00	3.96656667*	.39774415	.000	2.8604941	5.0726393
		1.00	1.81966667*	.39774415	.000	.7135941	2.9257393
		2.00	.67373333	.39774415	.443	-.4323393	1.7798059
		4.00	-.88093333	.39774415	.183	-1.9870059	.2251393
	4.00	.00	4.84750000*	.39774415	.000	3.7414274	5.9535726
		1.00	2.70060000*	.39774415	.000	1.5945274	3.8066726
		2.00	1.55466666*	.39774415	.002	.4485941	2.6607393
		3.00	.88093333	.39774415	.183	-.2251393	1.9870059
pistill	.00	1.00	-1.40070000*	.45017855	.020	-2.6525856	-.1488144
		2.00	-3.66893333*	.45017855	.000	-4.9208189	-2.4170478
		3.00	-5.57926667*	.45017855	.000	-6.8311522	-4.3273811
		4.00	-7.10053334*	.45017855	.000	-8.3524189	-5.8486478
	1.00	.00	1.40070000*	.45017855	.020	.1488144	2.6525856
		2.00	-2.26823333*	.45017855	.000	-3.5201189	-1.0163478
		3.00	-4.17856667*	.45017855	.000	-5.4304522	-2.9266811
		4.00	-5.69983333*	.45017855	.000	-6.9517189	-4.4479478
	2.00	.00	3.66893333*	.45017855	.000	2.4170478	4.9208189
		1.00	2.26823333*	.45017855	.000	1.0163478	3.5201189
		3.00	-1.91033333*	.45017855	.000	-3.1622189	-.6584478
		4.00	-3.43160000*	.45017855	.000	-4.6834856	-2.1797144
	3.00	.00	5.57926667*	.45017855	.000	4.3273811	6.8311522
		1.00	4.17856667*	.45017855	.000	2.9266811	5.4304522
		2.00	1.91033333*	.45017855	.000	.6584478	3.1622189

		4.00	-1.52126667*	.45017855	.009	-2.7731522	-.2693811
	4.00	.00	7.10053334*	.45017855	.000	5.8486478	8.3524189
		1.00	5.69983333*	.45017855	.000	4.4479478	6.9517189
		2.00	3.43160000*	.45017855	.000	2.1797144	4.6834856
		3.00	1.52126667*	.45017855	.009	.2693811	2.7731522
stamen3l	.00	1.00	-1.78160000*	.31690623	.000	-2.6628733	-.9003267
		2.00	-3.90320000*	.31690623	.000	-4.7844733	-3.0219267
		3.00	-5.43663333*	.31690623	.000	-6.3179067	-4.5553600
		4.00	-6.24376667*	.31690623	.000	-7.1250400	-5.3624933
	1.00	.00	1.78160000*	.31690623	.000	.9003267	2.6628733
		2.00	-2.12160000*	.31690623	.000	-3.0028733	-1.2403267
		3.00	-3.65503333*	.31690623	.000	-4.5363067	-2.7737600
		4.00	-4.46216667*	.31690623	.000	-5.3434400	-3.5808933
	2.00	.00	3.90320000*	.31690623	.000	3.0219267	4.7844733
		1.00	2.12160000*	.31690623	.000	1.2403267	3.0028733
		3.00	-1.53343333*	.31690623	.000	-2.4147067	-.6521600
		4.00	-2.34056667*	.31690623	.000	-3.2218400	-1.4592933
	3.00	.00	5.43663333*	.31690623	.000	4.5553600	6.3179067
		1.00	3.65503333*	.31690623	.000	2.7737600	4.5363067
		2.00	1.53343333*	.31690623	.000	.6521600	2.4147067
		4.00	-.80713333	.31690623	.089	-1.6884067	.0741400
	4.00	.00	6.24376667*	.31690623	.000	5.3624933	7.1250400
		1.00	4.46216667*	.31690623	.000	3.5808933	5.3434400
		2.00	2.34056667*	.31690623	.000	1.4592933	3.2218400
		3.00	.80713333	.31690623	.089	-.0741400	1.6884067
ASC	.00	1.00	-.06000	.06590	.892	-.2433	.1233
		2.00	-.35000*	.06590	.000	-.5333	-.1667
		3.00	-.96000*	.06590	.000	-1.1433	-.7767
		4.00	-1.00000*	.06590	.000	-1.1833	-.8167
	1.00	.00	.06000	.06590	.892	-.1233	.2433
		2.00	-.29000*	.06590	.000	-.4733	-.1067
		3.00	-.90000*	.06590	.000	-1.0833	-.7167
		4.00	-.94000*	.06590	.000	-1.1233	-.7567
	2.00	.00	.35000*	.06590	.000	.1667	.5333
		1.00	.29000*	.06590	.000	.1067	.4733
		3.00	-.61000*	.06590	.000	-.7933	-.4267
		4.00	-.65000*	.06590	.000	-.8333	-.4667
	3.00	.00	.96000*	.06590	.000	.7767	1.1433
		1.00	.90000*	.06590	.000	.7167	1.0833
		2.00	.61000*	.06590	.000	.4267	.7933
		4.00	-.04000	.06590	.974	-.2233	.1433
	4.00	.00	1.00000*	.06590	.000	.8167	1.1833
		1.00	.94000*	.06590	.000	.7567	1.1233
		2.00	.65000*	.06590	.000	.4667	.8333
		3.00	.04000	.06590	.974	-.1433	.2233

*. The mean difference is significant at the 0.05 level.

Appendix 1.8 Regression Analyses for Mean of Offspring x Midparent Floral-Traits in *Collinsia heterophylla* Norway population

Corolla Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring corolla length (mm)	18.0722872	1.34164133	100
midparent corolla length (mm)	17.5409816	1.35997766	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.776[a]	.602	.598	.85097109	.602	148.081	1	98	.000

a. Predictors: (Constant), midparent corolla length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	107.233	1	107.233	148.081	.000[a]
	Residual	70.967	98	.724		
	Total	178.200	99			

a. Predictors: (Constant), midparent corolla length (mm)

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	4.649	1.106		4.202	.000	2.453	6.844
midparent corolla length (mm)	.765	.063	.776	12.169	.000	.640	.890

a. Dependent Variable: mean offspring corolla length (mm)

Anther-Stigma Contact

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring anther-stigma contact	.3937	.39176	100
midparent anther-stigma contact	.3800	.44359	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.667[a]	.445	.439	.29340	.445	78.511	1	98	.000

a. Predictors: (Constant), midparent anther-stigma contact

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	6.758	1	6.758	78.511	.000[a]
	Residual	8.436	98	.086		
	Total	15.194	99			

a. Predictors: (Constant), midparent anther-stigma contact

b. Dependent Variable: mean offspring anther-stigma contact

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
1 (Constant)	.170	.039		4.387	.000	.093	.247
midparent anther-stigma contact	.589	.066	.667	8.861	.000	.457	.721

a. Dependent Variable: mean offspring anther-stigma contact

Stamen3 Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring stamen3 length (mm)	12.5872991	2.32303574	100
midparent stamen3 length (mm)	12.4984860	2.67491471	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.867[a]	.751	.749	1.16419500	.751	296.181	1	98	.000

a. Predictors: (Constant), midparent stamen3 length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	401.429	1	401.429	296.181	.000[a]
	Residual	132.824	98	1.355		
	Total	534.253	99			

a. Predictors: (Constant), midparent stamen3 length (mm)

b. Dependent Variable: mean offspring stamen3 length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	3.179	.559		5.686	.000	2.069	4.288
	midparent stamen3 length (mm)	.753	.044	.867	17.210	.000	.666	.840

a. Dependent Variable: mean offspring stamen3 length (mm)

Pistil Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring pistil length (mm)	12.5109965	2.25499562	100
midparent pistil length (mm)	12.3716631	2.24000520	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.937[a]	.878	.877	.79030852	.878	707.997	1	98	.000

a. Predictors: (Constant), midparent pistil length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	442.206	1	442.206	707.997	.000[a]
	Residual	61.210	98	.625		
	Total	503.416	99			

a. Predictors: (Constant), midparent pistil length (mm)

b. Dependent Variable: mean offspring pistil length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	.838	.446		1.880	.063	-.046	1.723
	midparent pistil length (mm)	.944	.035	.937	26.608	.000	.873	1.014

a. Dependent Variable: mean offspring pistil length (mm)

Stamen1 Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring stamen1 length (mm)	14.6219354	1.54703872	100
midparent stamen1 length (mm)	14.4388096	1.71809972	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.878[a]	.771	.769	.74382818	.771	330.245	1	98	.000

a. Predictors: (Constant), midparent stamen1 length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	182.718	1	182.718	330.245	.000[a]
	Residual	54.221	98	.553		
	Total	236.940	99			

a. Predictors: (Constant), midparent stamen1 length (mm)

b. Dependent Variable: mean offspring stamen1 length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	3.205	.633		5.066	.000	1.949	4.460
	midparent stamen1 length (mm)	.791	.044	.878	18.173	.000	.704	.877

a. Dependent Variable: mean offspring stamen1 length (mm)

Keel Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring keel length (mm)	16.6989770	1.23318998	100
midparent keel length (mm)	16.3389342	1.36861855	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.783[a]	.613	.609	.77103415	.613	155.249	1	98	.000

a. Predictors: (Constant), midparent keel length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	92.295	1	92.295	155.249	.000[a]
	Residual	58.260	98	.594		
	Total	150.555	99			

a. Predictors: (Constant), midparent keel length (mm)

b. Dependent Variable: mean offspring keel length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	5.172	.928		5.571	.000	3.330	7.014
	midparent keel length (mm)	.705	.057	.783	12.460	.000	.593	.818

a. Dependent Variable: mean offspring keel length (mm)

Banner Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring banner length (mm)	6.5511305	.38996974	100
midparent banner length (mm)	6.4627311	.54089581	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.518[a]	.268	.260	.33536584	.268	35.863	1	98	.000

a. Predictors: (Constant), midparent banner length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	4.033	1	4.033	35.863	.000[a]
	Residual	11.022	98	.112		
	Total	15.056	99			

a. Predictors: (Constant), midparent banner length (mm)

b. Dependent Variable: mean offspring banner length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	4.139	.404		10.243	.000	3.337	4.941
	midparent banner length (mm)	.373	.062	.518	5.989	.000	.250	.497

a. Dependent Variable: mean offspring banner length (mm)

Wing Width

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring wing width (mm)	9.2449967	1.14364635	100
midparent wing width (mm)	8.6940766	1.00709390	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.470[a]	.221	.213	1.01445250	.221	27.822	1	98	.000

a. Predictors: (Constant), midparent wing width (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	28.632	1	28.632	27.822	.000[a]
	Residual	100.853	98	1.029		
	Total	129.485	99			

a. Predictors: (Constant), midparent wing width (mm)

b. Dependent Variable: mean offspring wing width (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	4.602	.886		5.195	.000	2.844	6.361
	midparent wing width (mm)	.534	.101	.470	5.275	.000	.333	.735

a. Dependent Variable: mean offspring wing width (mm)

Appendix 1.9 Regression Analyses for Mean of Offspring x Midparent Floral-Traits in *Collinsia heterophylla* Chiltern population

Anther-Stigma Contact

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring ASC	.4627	.44149	100
midparent ASC	.4740	.47792	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.878[a]	.772	.769	.21203	.772	331.215	1	98	.000

a. Predictors: (Constant), midparent ASC

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	14.890	1	14.890	331.215	.000[a]
	Residual	4.406	98	.045		
	Total	19.296	99			

a. Predictors: (Constant), midparent ASC

b. Dependent Variable: mean offspring ASC

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	.078	.030		2.606	.011	.019	.137
	midparent ASC	.811	.045	.878	18.199	.000	.723	.900

a. Dependent Variable: mean offspring ASC

Stamen3 Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring stamen3 length (mm)	13.4747067	2.54764369	100
midparent stamen length (mm)	13.4983733	2.51906039	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.870[a]	.757	.754	1.26252900	.757	305.116	1	98	.000

a. Predictors: (Constant), midparent stamen length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	486.348	1	486.348	305.116	.000[a]
	Residual	156.210	98	1.594		
	Total	642.558	99			

a. Predictors: (Constant), midparent stamen length (mm)

b. Dependent Variable: mean offspring stamen3 length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	1.598	.692		2.311	.023	.226	2.970
	midparent stamen length (mm)	.880	.050	.870	17.468	.000	.780	.980

a. Dependent Variable: mean offspring stamen3 length (mm)

Pistil Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring pistil length (mm)	14.3305533	2.99774376	100
midparent pistil length (mm)	13.8022200	2.96738943	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.825[a]	.680	.677	1.70394493	.680	208.417	1	98	.000

a. Predictors: (Constant), midparent pistil length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	605.124	1	605.124	208.417	.000[a]
	Residual	284.536	98	2.903		
	Total	889.660	99			

a. Predictors: (Constant), midparent pistil length (mm)

b. Dependent Variable: mean offspring pistil length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	2.831	.815		3.476	.001	1.215	4.448
	midparent pistil length (mm)	.833	.058	.825	14.437	.000	.719	.948

a. Dependent Variable: mean offspring pistil length (mm)

Stamen1 Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring stamen1 length (mm)	16.2683356	1.71235646	100
midparent stamen1 length(mm)	16.1797600	2.08719980	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.704[a]	.495	.490	1.22256914	.495	96.212	1	98	.000

a. Predictors: (Constant), midparent stamen1 length(mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	143.806	1	143.806	96.212	.000[a]
	Residual	146.478	98	1.495		
	Total	290.284	99			

a. Predictors: (Constant), midparent stamen1 length(mm)

b. Dependent Variable: mean offspring stamen1 length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	6.925	.960		7.212	.000	5.020	8.831
	midparent stamen1 length(mm)	.577	.059	.704	9.809	.000	.461	.694

a. Dependent Variable: mean offspring stamen1 length (mm)

Keel Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring keel length (mm)	19.4862933	1.62579791	100
midparent keel length (mm)	19.1541600	1.95804650	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.492[a]	.242	.234	1.42265875	.242	31.291	1	98	.000

a. Predictors: (Constant), midparent keel length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	63.331	1	63.331	31.291	.000[a]
	Residual	198.348	98	2.024		
	Total	261.679	99			

a. Predictors: (Constant), midparent keel length (mm)

b. Dependent Variable: mean offspring keel length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	11.662	1.406		8.295	.000	8.872	14.452
	midparent keel length (mm)	.408	.073	.492	5.594	.000	.264	.553

a. Dependent Variable: mean offspring keel length (mm)

Banner Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring banner length (mm)	8.5494756	.68332723	100
midparent banner length	8.0949867	1.03596850	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.042[a]	.002	-.008	.68620219	.002	.172	1	98	.679

a. Predictors: (Constant), midparent banner length

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.081	1	.081	.172	.679[a]
	Residual	46.146	98	.471		
	Total	46.227	99			

a. Predictors: (Constant), midparent banner length

b. Dependent Variable: mean offspring banner length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	8.326	.543		15.326	.000	7.248	9.404
	midparent banner length	.028	.067	.042	.415	.679	-.104	.160

a. Dependent Variable: mean offspring banner length (mm)

Wing Width

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring wing width (mm)	12.6516378	1.33924412	100
midparent wing width (mm)	12.2411600	1.73366425	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.459[a]	.210	.202	1.19608411	.210	26.117	1	98	.000

a. Predictors: (Constant), midparent wing width (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	37.363	1	37.363	26.117	.000[a]
	Residual	140.200	98	1.431		
	Total	177.564	99			

a. Predictors: (Constant), midparent wing width (mm)

b. Dependent Variable: mean offspring wing width (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	8.314	.857		9.699	.000	6.613	10.015
	midparent wing width (mm)	.354	.069	.459	5.110	.000	.217	.492

a. Dependent Variable: mean offspring wing width (mm)

Corolla Length

Descriptive Statistics

	Mean	Std. Deviation	N
mean offspring corolla length (mm)	21.1916711	1.78603553	100
midparent corolla length (mm)	20.8895267	2.11863718	100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.531[a]	.282	.274	1.52129930	.282	38.454	1	98	.000

a. Predictors: (Constant), midparent corolla length (mm)

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	88.996	1	88.996	38.454	.000[a]
	Residual	226.806	98	2.314		
	Total	315.802	99			

a. Predictors: (Constant), midparent corolla length (mm)

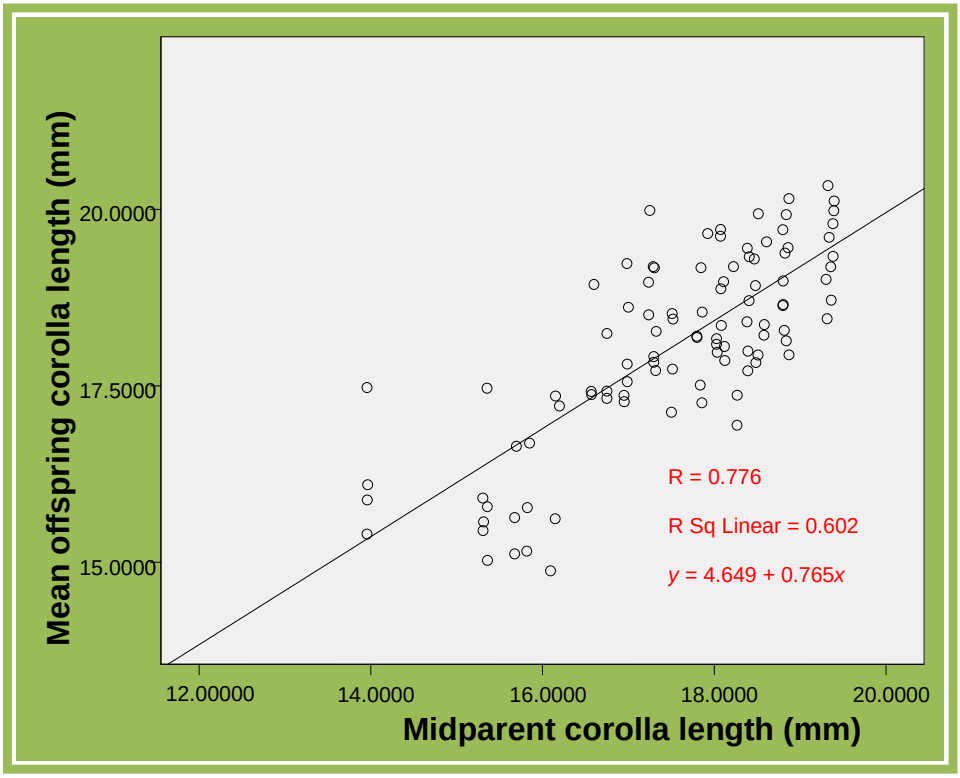
b. Dependent Variable: mean offspring corolla length (mm)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	11.843	1.515		7.816	.000	8.836	14.850
	midparent corolla length (mm)	.448	.072	.531	6.201	.000	.304	.591

a. Dependent Variable: mean offspring corolla length (mm)

<u>Appendix 1.10</u> Regression Analyses for Mean Offspring on Midparent Floral-Traits in *Collinsia heterophylla* Norway & Chiltern populations



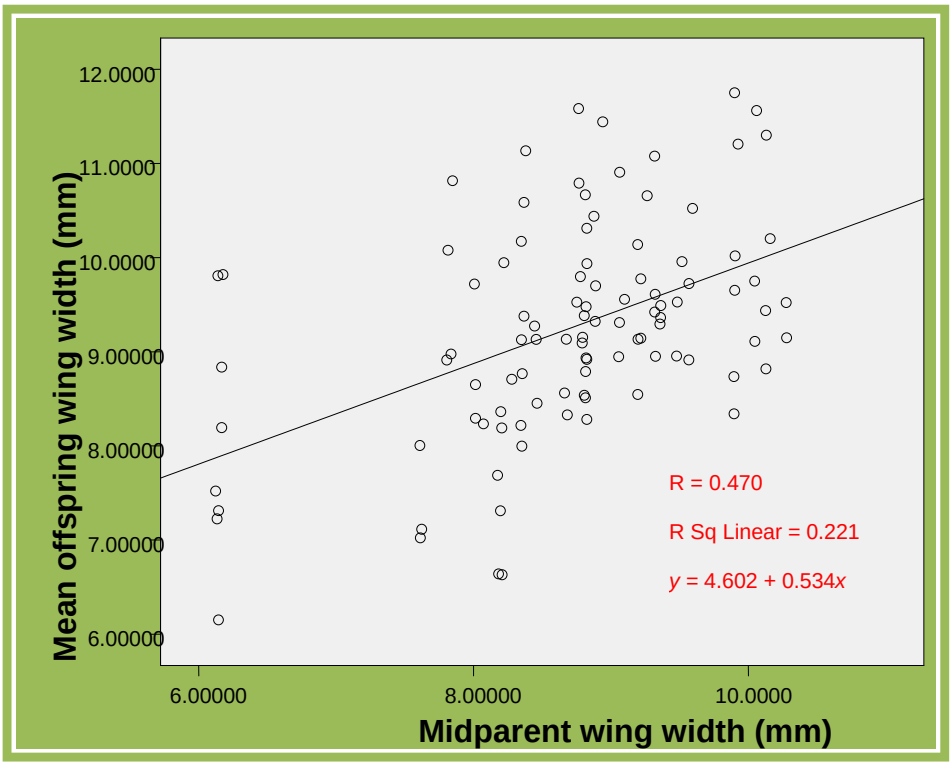
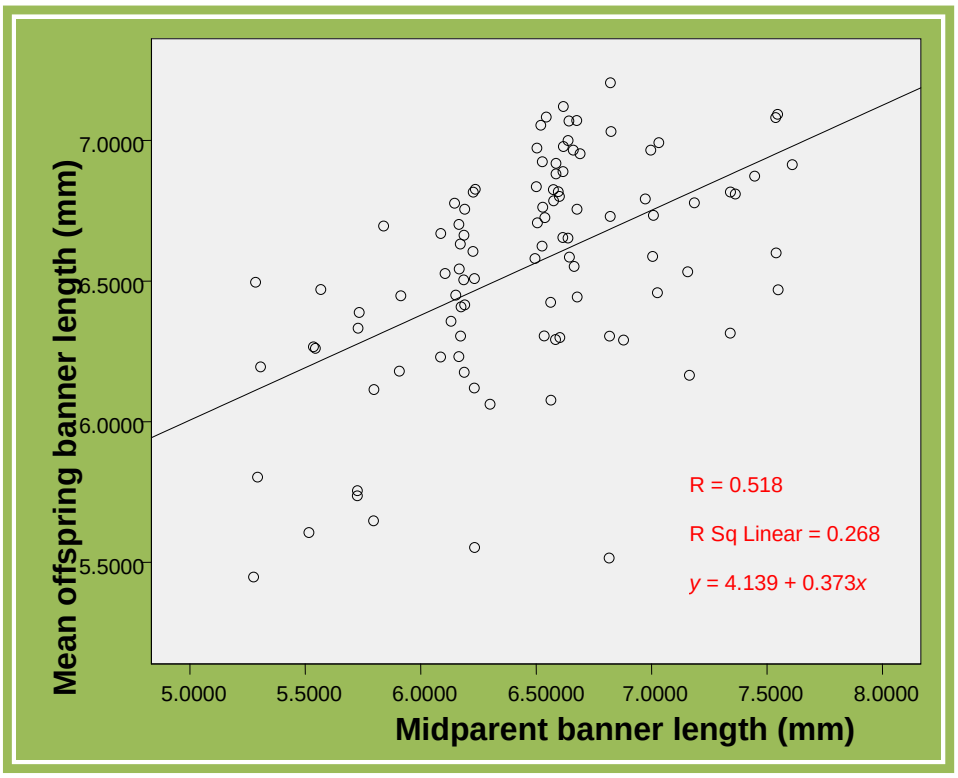


Figure 3.1 Simple Linear Regressions of **corolla length** and **wing width** mean offspring on midparent values in *Collinsia heterophylla* (Norway population). Regression equation (y = intercept + **slope** *x*); R^2 = coefficient of determination, R = correlation.



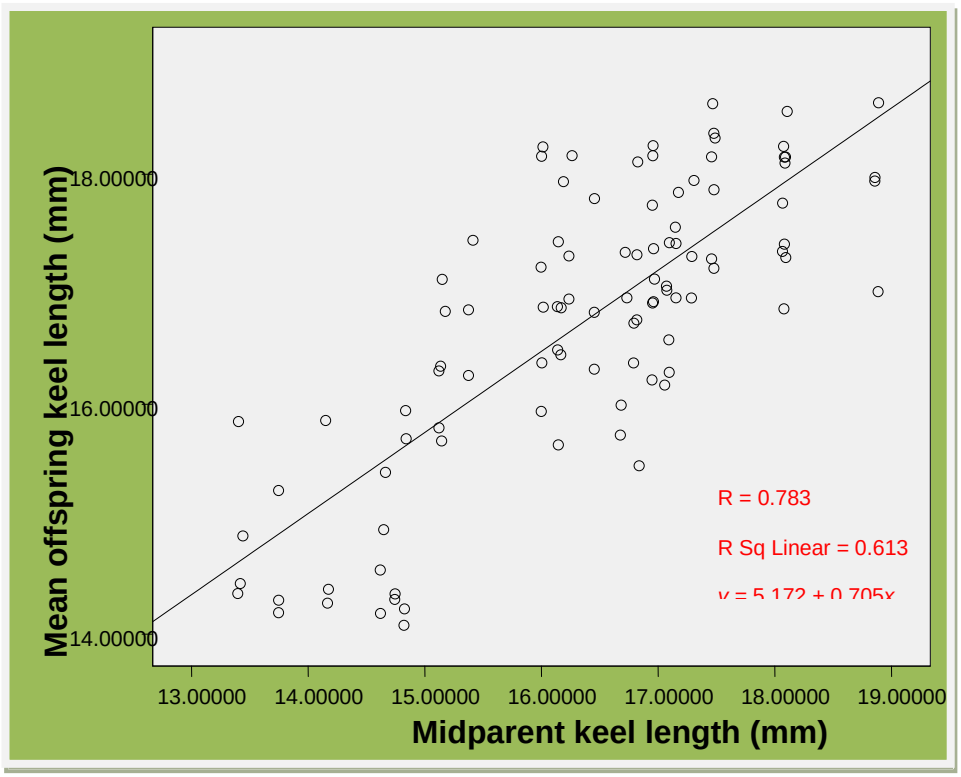
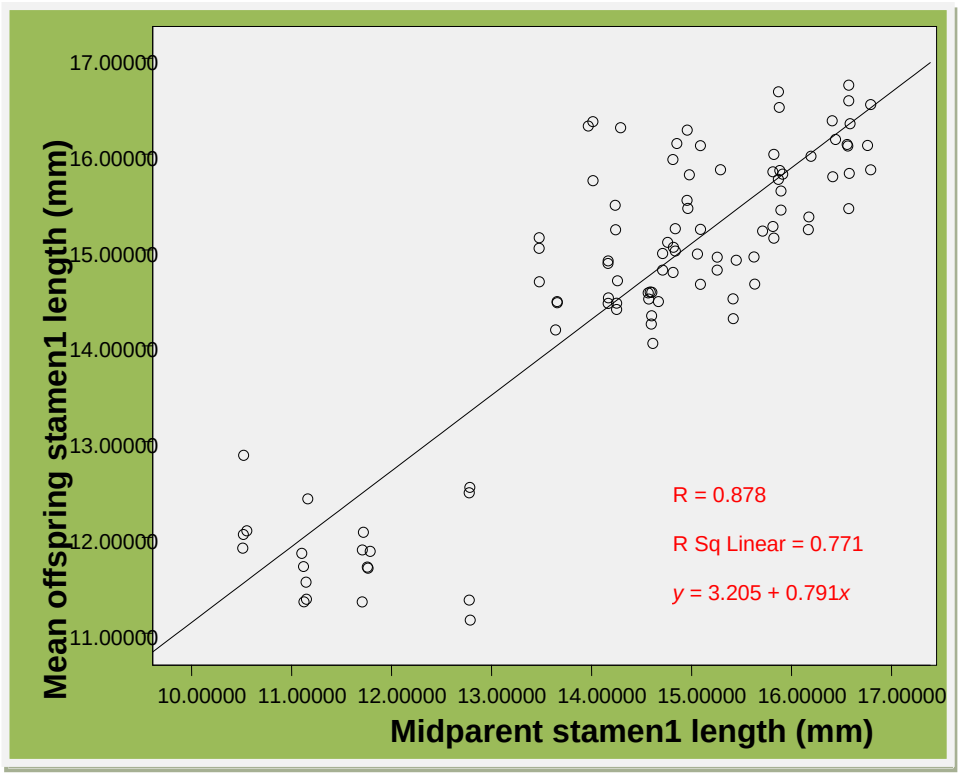


Figure 3.2 Simple Linear Regressions of **banner** and **keel length** mean offspring on midparent values in *Collinsia heterophylla* (Norway population). Regression equation (y = intercept + **slope** *x*); R^2 = coefficient of determination, R = correlation.



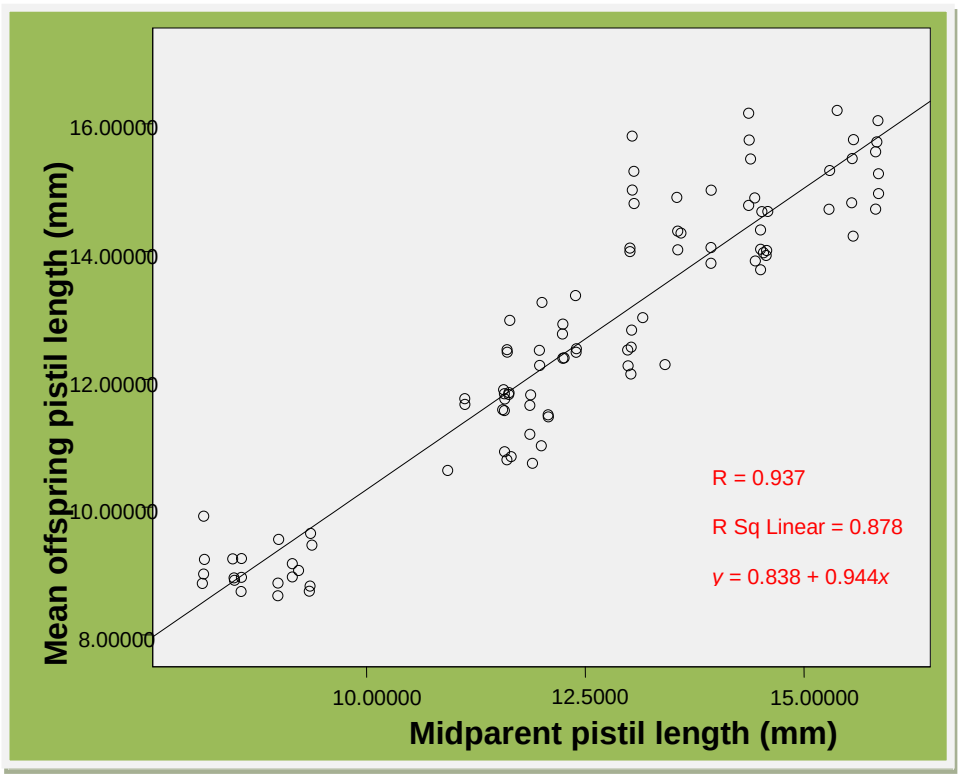


Figure 3.3 Simple Linear Regressions of **stamen1** and **pistil length** mean offspring on midparent values in *Collinsia heterophylla* (Norway population). Regression equation (y = intercept + **slope** *x*); R^2 = coefficient of determination, R = correlation.

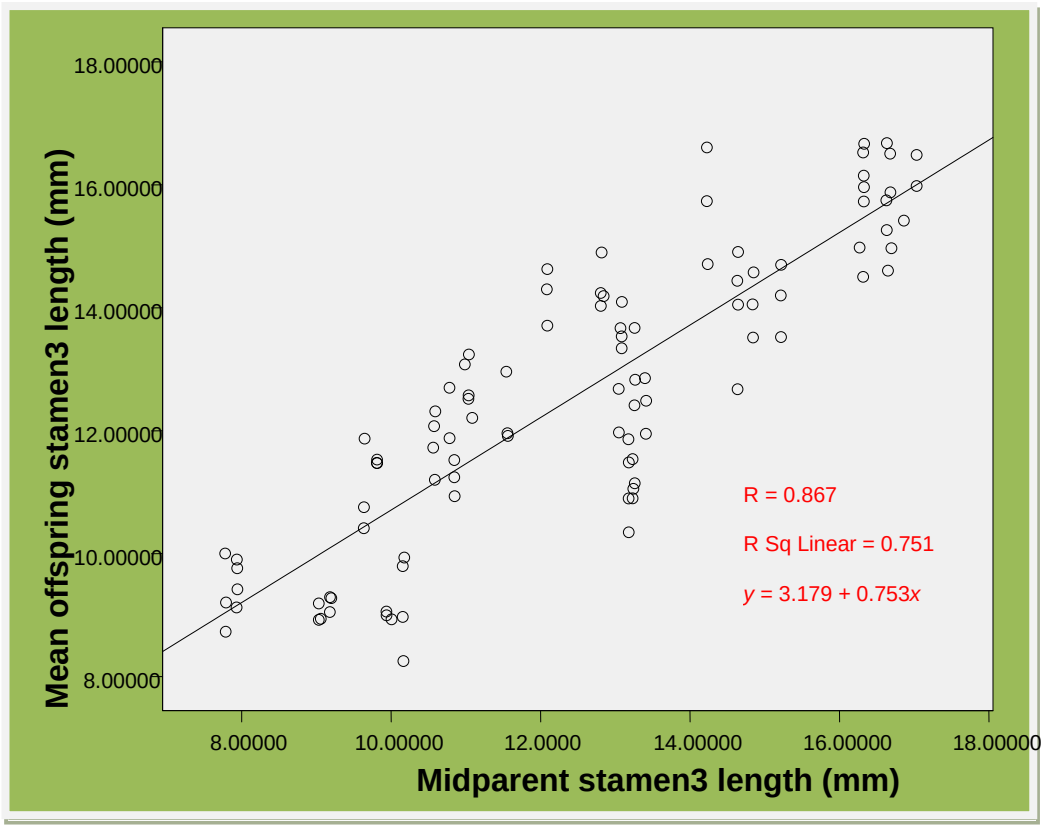
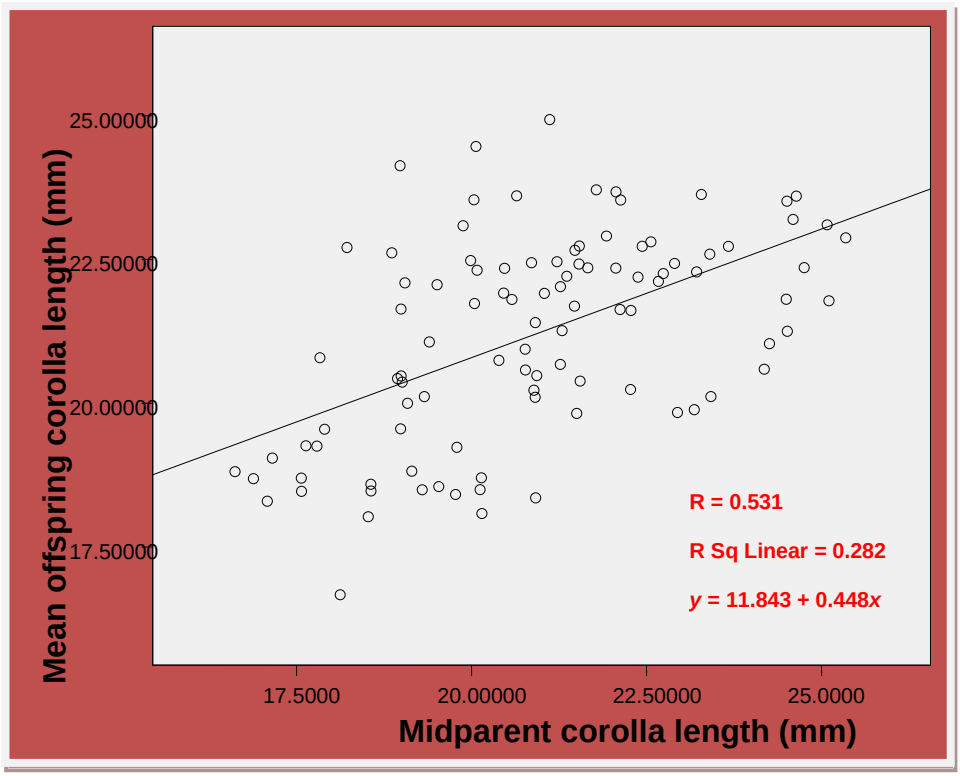


Figure 3.4 Simple Linear Regressions of **stamen 3 length** mean offspring on midparent values in *Collinsia heterophylla* (Norway population). Regression equation (y = intercept + **slope** *x*); R^2 = coefficient of determination, R = correlation.



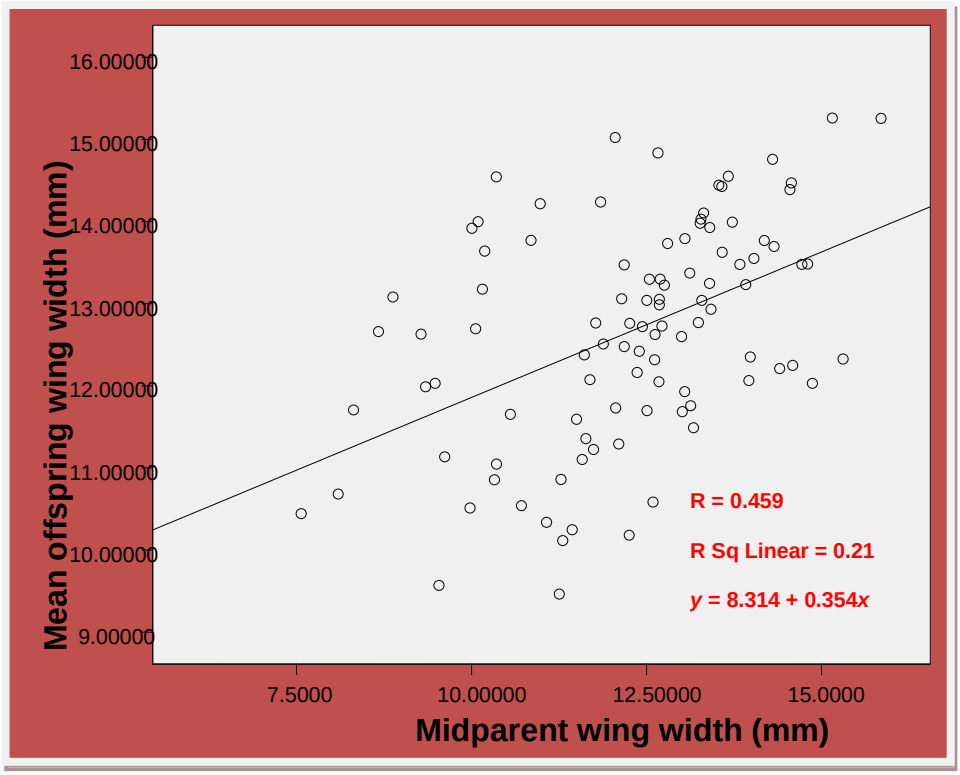
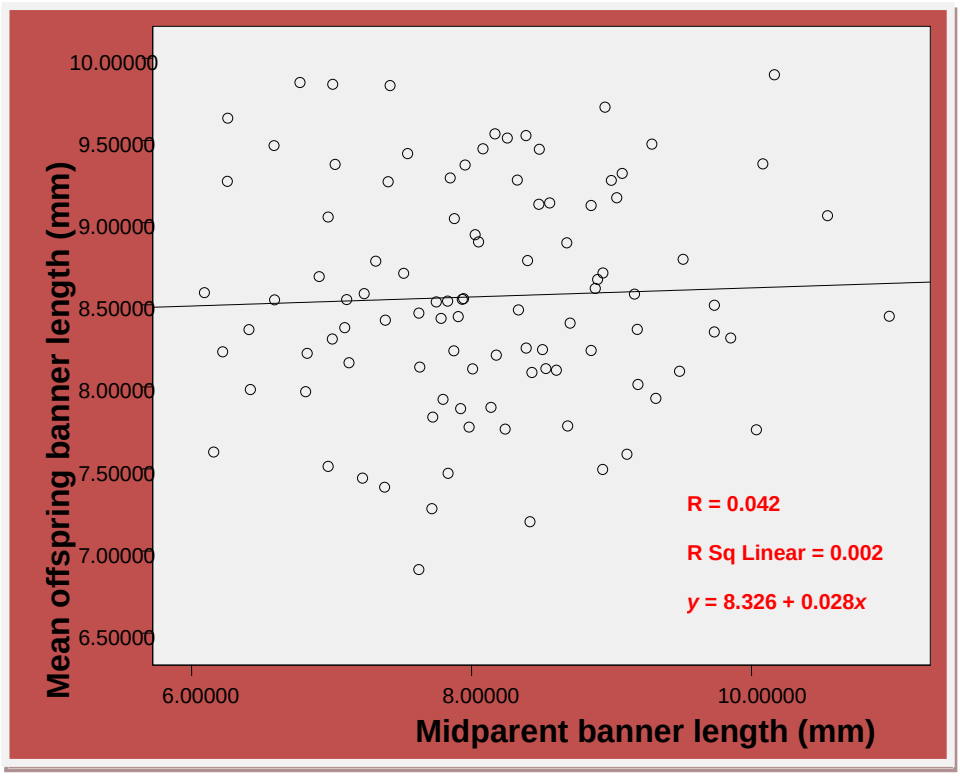


Figure 3.5 Simple Linear Regressions of **corolla length** and **wing width** mean offspring on midparent values in *Collinsia heterophylla* (Chiltern population). Regression equation (y = intercept + **slope***x*); R^2 = coefficient of determination, R = correlation.



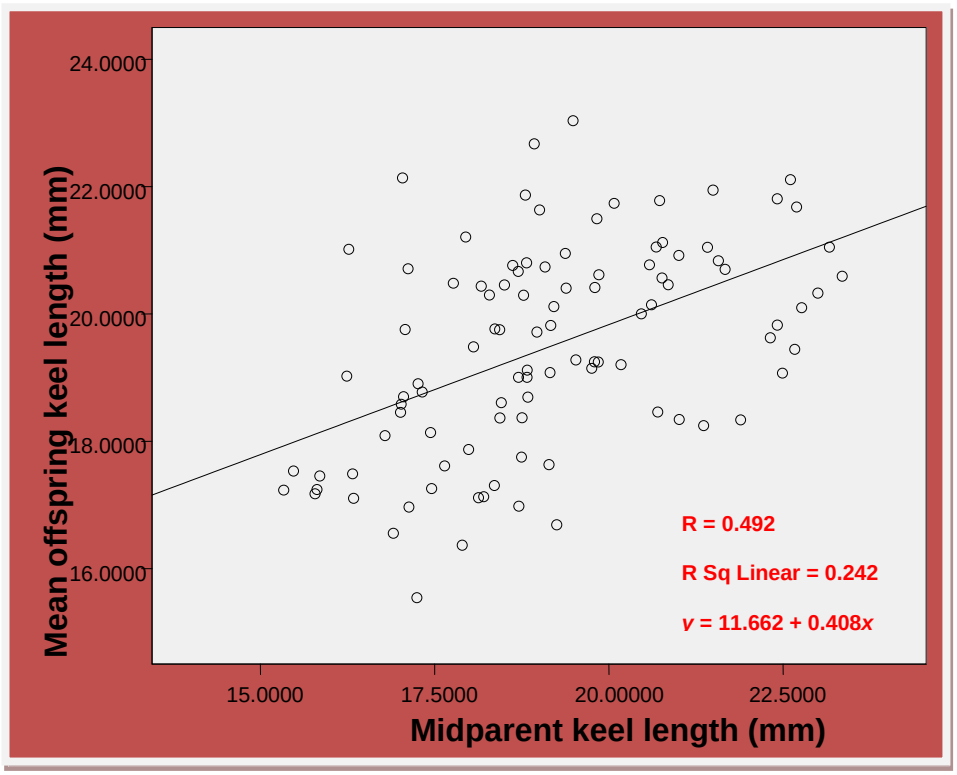
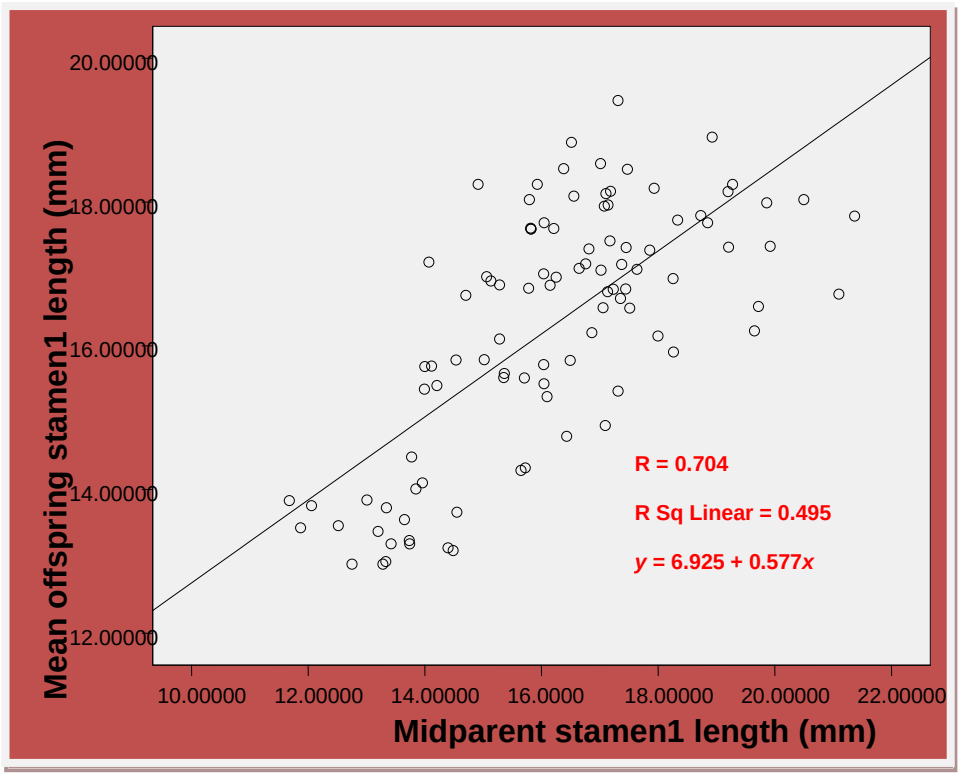


Figure 3.6 Simple Linear Regressions of **banner length** and **keel length** mean offspring on midparent values in *Collinsia heterophylla* (Chiltern population). Regression equation (y = intercept + **slope***x*); R^2 = coefficient of determination, R = correlation.



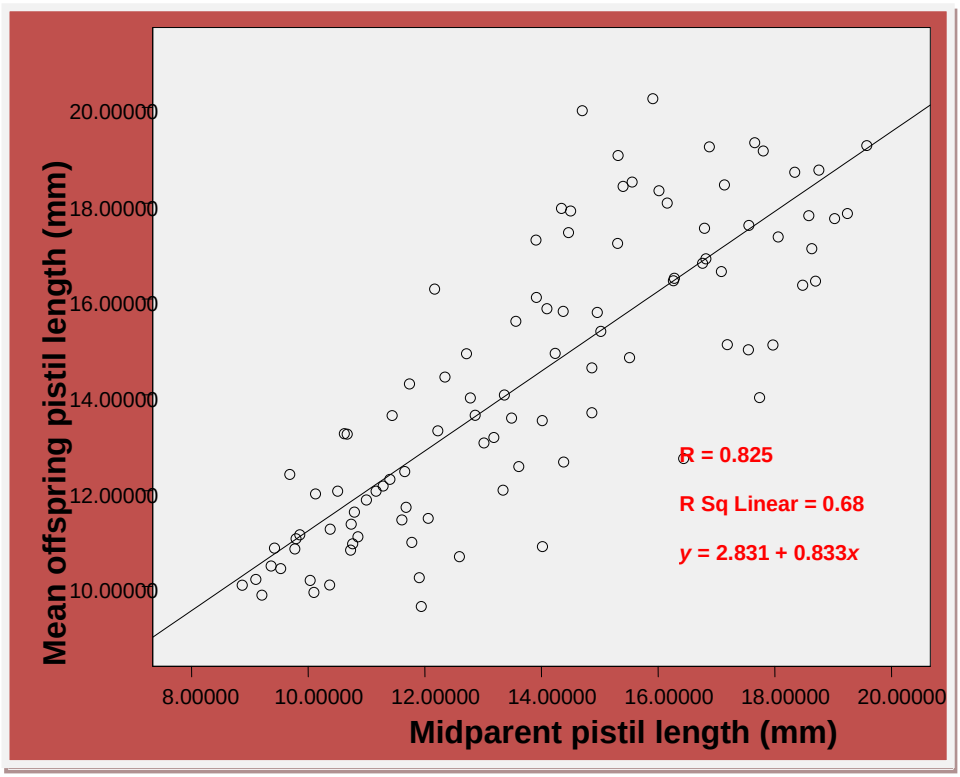


Figure 3.7 Simple Linear Regressions of **stamen 1** and **pistil length** mean offspring on midparent values in *Collinsia heterophylla* (Chiltern population). Regression equation (y = intercept + **slope***x*); R^2 = coefficient of determination, R = correlation.

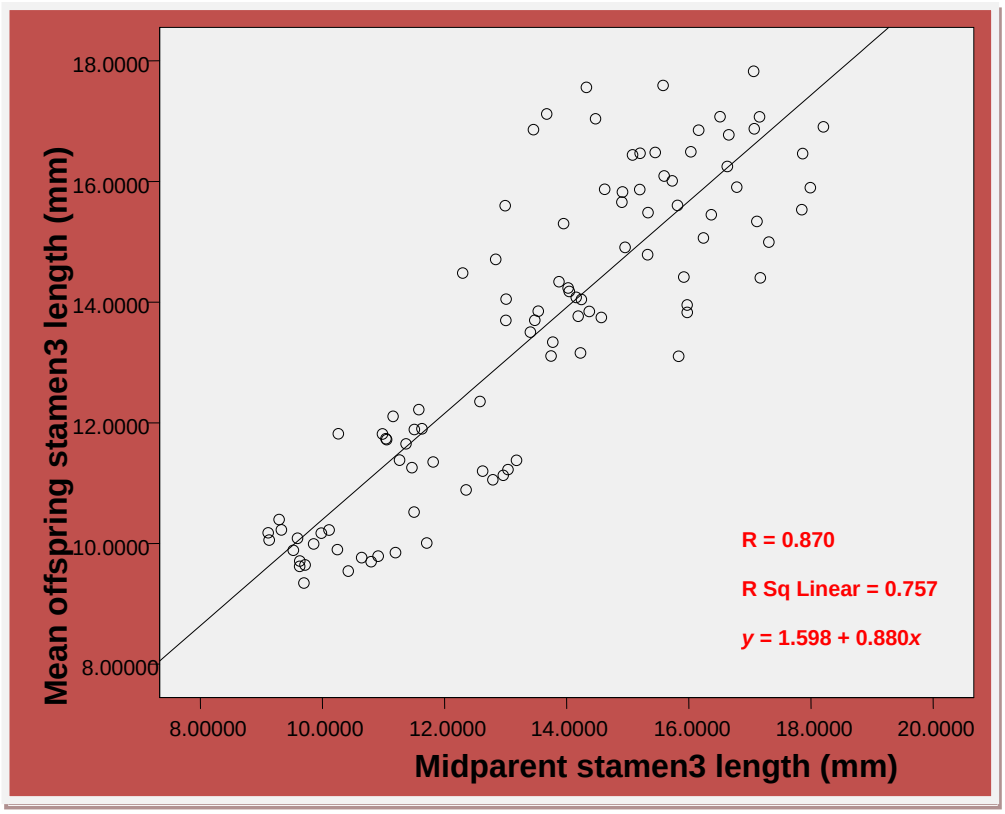


Figure 3.8 Simple Linear Regressions of **stamen 3 length** mean offspring on midparent values in *Collinsia heterophylla* (Chiltern population). Regression equation (y = intercept + **slope***x*); R^2 = coefficient of determination, R = correlation.

Appendix 2.0 Homogeneity and Normality Tests for Data in Chapter 4

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Hand-crossed	35.296	3	236	.000
Bagged	19.350	3	236	.000
Hand-selfed	38.962	3	236	.000
Emasculated	12.282	3	236	.000

Tests of Normality

	Population	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Hand-crossed (HC)	Chiltern	.128	120	.000	.948	120	.000
	Norway/American	.161	120	.000	.918	120	.000
Bagged (BG)	Chiltern	.094	120	.011	.953	120	.000
	Norway/American	.185	120	.000	.884	120	.000
Hand-selfed (HS)	Chiltern	.143	120	.000	.947	120	.000
	Norway/American	.161	120	.000	.931	120	.000
Emasculated (EM)	Chiltern	.186	120	.000	.912	120	.000
	Norway/American	.116	120	.000	.965	120	.003
Unmanipulated (UM)	Chiltern	.187	120	.000	.920	120	.000
	Norway/American	.180	120	.000	.940	120	.000

a. Lilliefors Significance Correction

Appendix 2.1 *Post Hoc* **Tests for Chiltern Population**

Multiple Comparisons

Dunnett T3

Dependent Variable	(I) Floral stages	(J) Floral stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Hand-crossed	stage 0	stage 1	-.75667*	.27210	.044	-1.4996	-.0138
		stage 2	-.96867*	.24755	.002	-1.6428	-.2945
		stage 3	-.06867	.22561	1.000	-.6822	.5448
	stage 1	stage 0	.75667*	.27210	.044	.0138	1.4996
		stage 2	-.21200	.29548	.977	-1.0160	.5920
		stage 3	.68800	.27736	.092	-.0684	1.4444
	stage 2	stage 0	.96867*	.24755	.002	.2945	1.6428
		stage 1	.21200	.29548	.977	-.5920	1.0160
		stage 3	.90000*	.25332	.005	.2106	1.5894
	stage 3	stage 0	.06867	.22561	1.000	-.5448	.6822
		stage 1	-.68800	.27736	.092	-1.4444	.0684
		stage 2	-.90000*	.25332	.005	-1.5894	-.2106
Bagged	stage 0	stage 1	1.36767*	.16694	.000	.9048	1.8305
		stage 2	1.67200*	.21617	.000	1.0696	2.2744
		stage 3	2.00033*	.17780	.000	1.5067	2.4940
	stage 1	stage 0	-1.36767*	.16694	.000	-1.8305	-.9048
		stage 2	.30433	.26089	.810	-.4069	1.0155
		stage 3	.63267*	.23010	.046	.0069	1.2584
	stage 2	stage 0	-1.67200*	.21617	.000	-2.2744	-1.0696
		stage 1	-.30433	.26089	.810	-1.0155	.4069
		stage 3	.32833	.26797	.774	-.4014	1.0580
	stage 3	stage 0	-2.00033*	.17780	.000	-2.4940	-1.5067
		stage 1	-.63267*	.23010	.046	-1.2584	-.0069
		stage 2	-.32833	.26797	.774	-1.0580	.4014
Hand-selfed	stage 0	stage 1	-.24533	.25594	.913	-.9425	.4519
		stage 2	-1.59100*	.30920	.000	-2.4381	-.7439
		stage 3	-.07933	.25409	1.000	-.7714	.6127
	stage 1	stage 0	.24533	.25594	.913	-.4519	.9425
		stage 2	-1.34567*	.33321	.001	-2.2539	-.4374
		stage 3	.16600	.28282	.992	-.6030	.9350
	stage 2	stage 0	1.59100*	.30920	.000	.7439	2.4381
		stage 1	1.34567*	.33321	.001	.4374	2.2539
		stage 3	1.51167*	.33180	.000	.6071	2.4162
	stage 3	stage 0	.07933	.25409	1.000	-.6127	.7714
		stage 1	-.16600	.28282	.992	-.9350	.6030
		stage 2	-1.51167*	.33180	.000	-2.4162	-.6071
Emasculated	stage 0	stage 1	.02133	.17986	1.000	-.4703	.5130
		stage 2	1.89900*	.31422	.000	1.0362	2.7618
		stage 3	1.97700*	.31320	.000	1.1171	2.8369
	stage 1	stage 0	-.02133	.17986	1.000	-.5130	.4703
		stage 2	1.87767*	.29299	.000	1.0650	2.6903
		stage 3	1.95567*	.29189	.000	1.1461	2.7652
	stage 2	stage 0	-1.89900*	.31422	.000	-2.7618	-1.0362
		stage 1	-1.87767*	.29299	.000	-2.6903	-1.0650
		stage 3	.07800	.38934	1.000	-.9807	1.1367
	stage 3	stage 0	-1.97700*	.31320	.000	-2.8369	-1.1171
		stage 1	-1.95567*	.29189	.000	-2.7652	-1.1461
		stage 2	-.07800	.38934	1.000	-1.1367	.9807

*. The mean difference is significant at the 0.05 level.

Appendix 2.2 *Post Hoc* **Tests for Norway Population**

Multiple Comparisons

Dunnett T3

Dependent Variable	(I) Floral stages	(J) Floral stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Hand-crossed	stage0	stage1	.20067	.11129	.373	-.1031	.5044
		stage2	1.74400*	.09290	.000	1.4914	1.9966
		stage3	2.27667*	.08449	.000	2.0468	2.5066
	stage1	stage0	-.20067	.11129	.373	-.5044	.1031
		stage2	1.54333*	.11299	.000	1.2352	1.8514
		stage3	2.07600*	.10617	.000	1.7852	2.3668
	stage2	stage0	-1.74400*	.09290	.000	-1.9966	-1.4914
		stage1	-1.54333*	.11299	.000	-1.8514	-1.2352
		stage3	.53267*	.08671	.000	.2966	.7687
	stage3	stage0	-2.27667*	.08449	.000	-2.5066	-2.0468
		stage1	-2.07600*	.10617	.000	-2.3668	-1.7852
		stage2	-.53267*	.08671	.000	-.7687	-.2966
Bagged	stage0	stage1	2.37667*	.13087	.000	2.0206	2.7328
		stage2	3.13200*	.14436	.000	2.7383	3.5257
		stage3	3.55500*	.11073	.000	3.2535	3.8565
	stage1	stage0	-2.37667*	.13087	.000	-2.7328	-2.0206
		stage2	.75533*	.15269	.000	.3398	1.1708
		stage3	1.17833*	.12139	.000	.8471	1.5096
	stage2	stage0	-3.13200*	.14436	.000	-3.5257	-2.7383
		stage1	-.75533*	.15269	.000	-1.1708	-.3398
		stage3	.42300*	.13583	.018	.0510	.7950
	stage3	stage0	-3.55500*	.11073	.000	-3.8565	-3.2535
		stage1	-1.17833*	.12139	.000	-1.5096	-.8471
		stage2	-.42300*	.13583	.018	-.7950	-.0510
Hand-selfed	stage0	stage1	-.08933	.14815	.991	-.4939	.3153
		stage2	1.43267*	.11167	.000	1.1289	1.7364
		stage3	1.62133*	.10350	.000	1.3391	1.9036
	stage1	stage0	.08933	.14815	.991	-.3153	.4939
		stage2	1.52200*	.14314	.000	1.1300	1.9140
		stage3	1.71067*	.13686	.000	1.3341	2.0873
	stage2	stage0	-1.43267*	.11167	.000	-1.7364	-1.1289
		stage1	-1.52200*	.14314	.000	-1.9140	-1.1300
		stage3	.18867	.09619	.281	-.0732	.4505
	stage3	stage0	-1.62133*	.10350	.000	-1.9036	-1.3391
		stage1	-1.71067*	.13686	.000	-2.0873	-1.3341
		stage2	-.18867	.09619	.281	-.4505	.0732
Emasculated	stage0	stage1	-.14467	.14176	.887	-.5308	.2415
		stage2	.81167*	.15079	.000	.4002	1.2231
		stage3	1.25600*	.13246	.000	.8956	1.6164
	stage1	stage0	.14467	.14176	.887	-.2415	.5308
		stage2	.95633*	.16562	.000	.5059	1.4068
		stage3	1.40067*	.14913	.000	.9950	1.8063
	stage2	stage0	-.81167*	.15079	.000	-1.2231	-.4002
		stage1	-.95633*	.16562	.000	-1.4068	-.5059
		stage3	.44433*	.15773	.039	.0148	.8738
	stage3	stage0	-1.25600*	.13246	.000	-1.6164	-.8956
		stage1	-1.40067*	.14913	.000	-1.8063	-.9950
		stage2	-.44433*	.15773	.039	-.8738	-.0148

*. The mean difference is significant at the 0.05 level.

Appendix 3.0 Recipe for the Growth medium (Hoekstra medium)

Pollen germinating Hoekstra medium for *Collinsia heterophylla*

Medium (50 ml):

7.4 mg $Ca(OH)_2$

konc. H_3PO_4 or some other acid

5.0 mg H_3BO_3

Mix $Ca(OH)_2$ with distilled water. Use the acid to adjust the pH to 6.8. (Dilute the acid and take it slow!) Add H_3BO_3.

Prepared medium for pollen germination (20-25 samples):

5 ml medium

0.5 g sucrose

50 mg agarose

- Mix medium, sucrose and agarose.
- Heat until you see bubbles (use moderate heat).
- Put microscopic slides in a plastic box (with a wet filter paper).
- Pour 2-3 droplets on to each of the microscopic slides. Avoid a draft in the room, since this will damage the medium.
- Add pollen from 2-3 flowers. Make sure the pollen grains are not kept too close together.
- Germinate in a dark chamber at a steady temperature, e.g. 22°C. leave for 1.5 hours.
- Terminate pollen growth by adding concentrated glycerol.
- Add a covering glass and samples are ready to measure.
- Store the sample in the fridge. They can be stored for several weeks.

Appendix 3.1 Preparation of Aniline Blue Aqueous Stain 0.5% aqueous:

Dissolve 0.5 g aniline blue in 50 ml DI water, then dilute to 100 ml.

Filter if necessary (stain for algae and fungi)

Preparation of Buffer Solutions

Buffers are typically mixtures of a weak acid and the salt of the acid or a weak base and its salt. This combination is called a conjugate acid-base pair and it will resist changes in pH upon addition of small amounts of acid or base. Recipe for the buffer used is as follow:

pH 7: Prepare 0.10 M Potassium phosphate monobasic (KH_2PO_4) solution by dissolving 3.40 g in 250 ml distilled water. Prepare 0.20 M sodium hydroxide solution by dissolving 0.8 g in 100 ml distilled water. Mix 250 ml of the 0.10 M Potassium phosphate solution and 73 ml of 0.2 M sodium hydroxide solution then dilute to 500 ml.

Appendix 3.2 Homogeneity and Normality Tests for Data in Chapter 5

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Norway population pollen-tube length (mm)	25.034	1	88	.000
Chiltern population pollen-tube length (mm)	33.120	1	88	.000

Tests of Normality

	Germination type	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Chiltern pollen tube length (mm)	in-vitro	.141	50	.014	.932	50	.006
	in-vivo	.103	40	.200*	.970	40	.369
Norway pollen tube length (mm)	in-vitro	.127	50	.043	.950	50	.033
	in-vivo	.115	40	.198	.946	40	.057

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

Appendix 3.3 *Post hoc* Dunnett T3 Tests for Data in Chapter 5

Multiple Comparisons

Mean Pollen-tube length (mm)
Dunnett T3

(I) Pollination Treatment	(J) Pollination Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Norway flower selfed	Chiltern flower selfed	-.4626000*	.0329259	.000	-.555107	-.370093
	Chiltern flower x Norway pollen	-.1515000*	.0376638	.003	-.258014	-.044986
	Norway flower x Chiltern pollen	-.7848500*	.0549532	.000	-.942454	-.627246
Chiltern flower selfed	Norway flower selfed	.4626000*	.0329259	.000	.370093	.555107
	Chiltern flower x Norway pollen	.3111000*	.0448521	.000	.186818	.435382
	Norway flower x Chiltern pollen	-.3222500*	.0601083	.000	-.491021	-.153479
Chiltern flower x Norway pollen	Norway flower selfed	.1515000*	.0376638	.003	.044986	.258014
	Chiltern flower selfed	-.3111000*	.0448521	.000	-.435382	-.186818
	Norway flower x Chiltern pollen	-.6333500*	.0628288	.000	-.808661	-.458039
Norway flower x Chiltern pollen	Norway flower selfed	.7848500*	.0549532	.000	.627246	.942454
	Chiltern flower selfed	.3222500*	.0601083	.000	.153479	.491021
	Chiltern flower x Norway pollen	.6333500*	.0628288	.000	.458039	.808661

*. The mean difference is significant at the 0.05 level.